Nahrungsmittelbestandteile als Modulatoren der neuralen Entwicklung: Rolle des AhR

Inaugural-Dissertation

zur

Erlangung des Doktorgrades der

Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Oktober 2009

Angefertigt am Institut für umweltmedizinische Forschung (IUF)
an der Heinrich-Heine Universität Düsseldorf gGmbH

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Ellen Fritsche

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Tag der mündlichen Prüfung: 15.12.2009

Meinen Eltern

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1. Einleitung

1.1 Der Arylhydrocarbonrezeptor (AhR) und seine Rolle in der neuralen Entwicklung

1.1.1 Allgemeines

Im Jahr 1976 wurde der Arylhydrocarbon-Rezeptor (Ah-Rezeptor, AhR) von Poland und Mitarbeitern entdeckt (Poland et al. 1976). Der AhR ist ein durch Liganden aktivierter Transkriptionsfaktor und gehört zur Familie der *basic-Helix-Loop-Helix*-Per-Arnt-Sim-(bHLH/PAS-) Proteine. Er wird nahezu ubiquitär in vielen Geweben, unterschiedlich stark exprimiert (Okey et al. 1994).

Im inaktiven Zustand befindet sich der AhR im Zytoplasma, wo er unter anderem an zwei Moleküle des Hitzeschockproteins 90 (hsp90) und an das AhR-interagierende-Protein (AIP) gebunden ist (auch bekannt als XAP2 oder ARA9) (Chen and Perdew 1994;Ma and Whitlock, Jr. 1997;Perdew 1988). Nach Bindung des Liganden wandert der AhR-Komplex in den Kern, wo er nach Abspaltung von hsp90 und AIP mit einem zweiten bHLH-Protein, dem AhR-Nuclear-Translocator (ARNT) heterodimerisiert. Ein 1999 identifiziertes Protein, der Ah-Rezeptor-Repressor (AhRR), kann die Funktion des AhR hemmen, indem es selbst mit ARNT dimerisiert und damit die Bindung des ARNT an den AhR unterdrückt (Mimura et al. 1999). AhR und ARNT besitzen Domänen für die Bindung an spezifische Sequenzen in der Promoterregion der AhR-Zielgene (Reyes et al. 1992). Diese kurzen DNS-Sequenzen (5`-TGCGTG-3`) werden als *Dioxin Response Elements* (DRE) oder *Xenobiotic Response Elements* (XRE) bezeichnet (Rowlands and Gustafsson 1997). Die Kopplung des AhR-ARNT-Ligand-Komplexes an das DRE führt zur Initiation der Transkription von mRNS entsprechender Zielgene. Eine schematische Übersicht der AhR-Signalkaskade ist in Abb. 1 dargestellt.

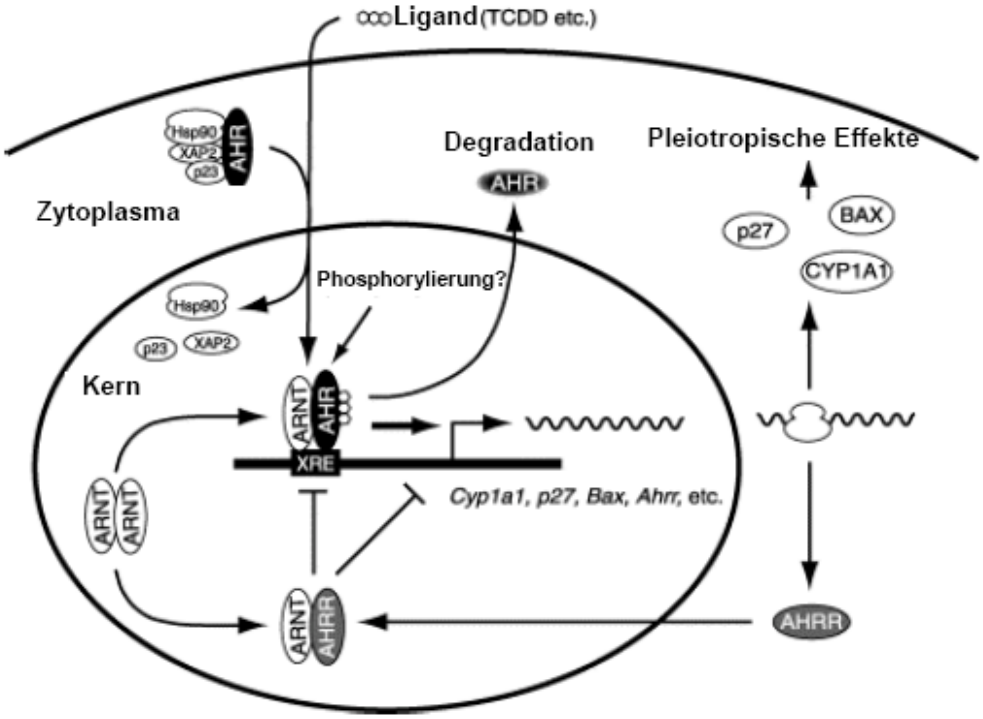


Abbildung 1: AhR-Signalkaskade modifiziert nach Mimura und Fujii-Kuriyama (2003). Erläuterung im Text

Die Mehrheit der Genprodukte dieser Zielgene wie die Cytochrom P450 Monooxygenasen (CYP) 1A1, 1A2 oder 1B1, UDP-Glukuronosyltransferasen, NAD(P)H-Quinon-Oxidoreduktase, Aldehyd-3-Dehydrogenase und Gluthathion-S-Transferase π1 (Aoki et al. 1992;Bock et al. 1990;Dunn et al. 1988;Jaiswal et al. 1988;Nebert and Gonzalez 1987) sind am Fremdstoffmetabolismus beteiligt.

Darüber hinaus gibt es aber auch Gene, denen keine direkte Rolle im Fremdstoffmetabolismus zugewiesen werden kann, die aber dennoch durch den AhR beeinflusst werden. Dazu gehören verschiedene Zytokine wie Plasminogen-Aktivator-Inhibitor (PAI) 2 und Interleukin (IL)-1β (Sutter et al. 1991;Vogel et al. 1997), Wachstumsfaktoren z.B. TGFα und β (Gaido et al. 1992) und Protoonkogene wie c-fos und c-jun (Puga et al. 1992). Die Regulationsmechanismen der Induktion oder Repression dieser Zielgene im weiteren Sinn sind noch nicht vollständig aufgeklärt (Haarmann-Stemmann et al. 2009). Auch werden AhR-Zielgene häufig Zelltyp-spezifisch reguliert (Shimada et al. 2003), was sogar dazu führen kann, dass eine AhR-Aktivierung in unterschiedlichen Zellmodellen zu gegensätzliche Effekten führt (Jin et al. 2004;Marlowe and Puga 2005;Wong et al. 2009).

Studien an Ah-Rezeptor-defizienten Mäusen bestätigten, dass der AhR über die Regulierung des Fremdstoffmetabolismus hinaus eine bedeutende Rolle in physiologischen Prozessen wie Zellproliferation und –differenzierung, der normalen Entwicklung und Funktion von Organen und dem Erhalt der Gewebshomöostase spielt (Fernandez-Salguero et al. 1996).

Das Fehlen des AhR hatte Herzhypertrophie (Lund et al. 2006), Immunsuppression (Fernandez-Salguero et al. 1995), Hautveränderungen (Fernandez-Salguero et al. 1997) und Gewebsveränderungen in der Leber (Fernandez-Salguero et al. 1997;Schmidt et al. 1996) in den entsprechenden Mäusen zur Folge. Daneben kam es zu Störungen während der Reproduktion und Entwicklung. Es zeigte sich, dass weibliche AhR-null Mäuse eine deutlich geringere Anzahl von Jungen gebären und deren Überlebensrate während der Säugezeit reduziert ist (Abbott et al. 1999). Zusätzlich ist in AhR-defizienten Mäusen der *Ductus venosus* in der Leber nach der Geburt nicht geschlossen (Lahvis et al. 2000). Dies führt dazu, dass der Leberkreislauf umgangen wird und das Blut von der Pfortader direkt in die Hohlvene gelangt. Es kommt zu keiner Entgiftung von Schadstoffen durch die Leber.

1.1.2 AhR und Entwicklungsneurotoxizität

Die Struktur des AhR ist evolutionär stark konserviert und Homologe zum Säuger-AhR konnten bereits in verschiedenen Invertebraten und Fischen nachgewiesen werden. Es zeigte sich, dass ein Fehlen oder der Funktionsverlust des AhR auch dort zu Entwicklungsdefekten besonders innerhalb des neuralen Systems führt. In *C. elegans* Mutanten mit nicht funktionsfähigem AhR kommt es, ausgelöst durch Veränderungen in der Genexpression, zu Defekten in der neuronalen Differenzierung. Im Einzelnen konnten Störungen in der Migration, Art und Anzahl der Neuritenverzweigungen und der Differenzierung von GABAergen Motorneuronen festgestellt werden (Huang et al. 2000;Qin et al. 2006;Qin and Powell-Coffman 2004). Ein ähnlicher Effekt wurde in *Drosophila melanogaster* anhand von Mutanten des AhR-Homologs Spineless beschrieben (Kim et al. 2006). In Mutanten mit nicht funktionsfähigem AhR kam es zu Veränderungen im Differenzierungsgrad und der Anzahl der Neuriten der sensorischen Neuronen. Interessanter Weise scheint der AhR in Invertebraten für seine physiologische Funktion keine Aktivierung durch einen Liganden zu benötigen (Emmons et al. 2007;Qin and Powell-Coffman 2004). Auch bei Wirbeltieren gibt es durch Studien am Modellorganismus Zebrafisch (*Danio rerio*) Hinweise auf eine essentielle Funktion des AhR während der neuralen Entwicklung. Die Exposition mit TCDD, einem der potentesten AhR-Liganden, während des Larvenstadiums hat eine deutlich Reduzierung der Neuronenzahl und damit des Gehirnvolumens zur Folge, die von einer verminderten Expression der beiden in der Entwicklung eine Schlüsselrolle spielenden Gene *neurogenin* und *sonic hedgehog* begleitet wird (Hill et al. 2003).

Um in Bezug auf die Rolle des AhR in der neuralen Entwicklung des Säugers nicht zuviel vorwegzunehmen verweise ich an dieser Stelle auf Publikation 2.3 im Hauptteil meiner Dissertation und wende mich im folgenden Abschnitt der wichtigen Frage nach möglichen exogenen und endogenen Aktivatoren des AhR zu.

1.2 AhR-Agonisten und Antagonisten

Eine Vielzahl von Studien in den letzten Jahrzehnten zeigte, dass der AhR - untypisch für Liganden-regulierte Rezeptoren - in der Lage ist, strukturell sehr unterschiedliche Substanzen zu binden. Es gibt zwei große Kategorien: (1) die synthetischen Liganden, die meist als Folge anthrophogener Aktivitäten entstehen und in der Umwelt weit verbreitet sind, und (2) die natürlichen Liganden, die größtenteils über die Nahrung in den Organismus gelangen oder endogen im Körper gebildet werden.

Mit den Wirkungen der klassischen synthetischen AhR-Liganden auf die neurale Entwicklung beschäftigt sich Publikation 2.3, wohingegen in Publikation 2.6 die Effekte natürlicher AhR-Liganden untersucht werden.

1.2.1 Klassische synthetische AhR-Liganden

Struktur-Aktivitäts-Untersuchungen mit polyaromatischen Kohlenwasserstoffen (PAK) und halogenierten aromatischen Kohlenwasserstoffen (HAK) ergaben, dass Liganden mit hoher Bindungsaffinität zumeist aromatische, planare und relativ hydrophobe Verbindungen sind. Zu ihnen gehören die synthetischen AhR-Liganden polychlorierte Dibenzodioxine und Dibenzofurane (PCDF), polychlorierte und polybromierte Biphenyle (PCB bzw. PBDE), Benzo[a]pyren (B[a]p) und 3-Methylcholanthren (3-MC), wobei die Verbindungen mit der höchsten Affinität zum AhR die höchste Toxizität aufweisen (Poland and Knutson 1982;Safe 1990;Whitlock, Jr. 1990). Der stärkste bisher bekannte synthetische Ligand des AhR ist das 2,3,7,8- Tetrachlordibenzo-p-dioxin (Safe 1984). In Abbildung 2 ist eine Übersicht über die Struktur dieser wichtigsten „klassischen“ AhR-Liganden dargestellt.

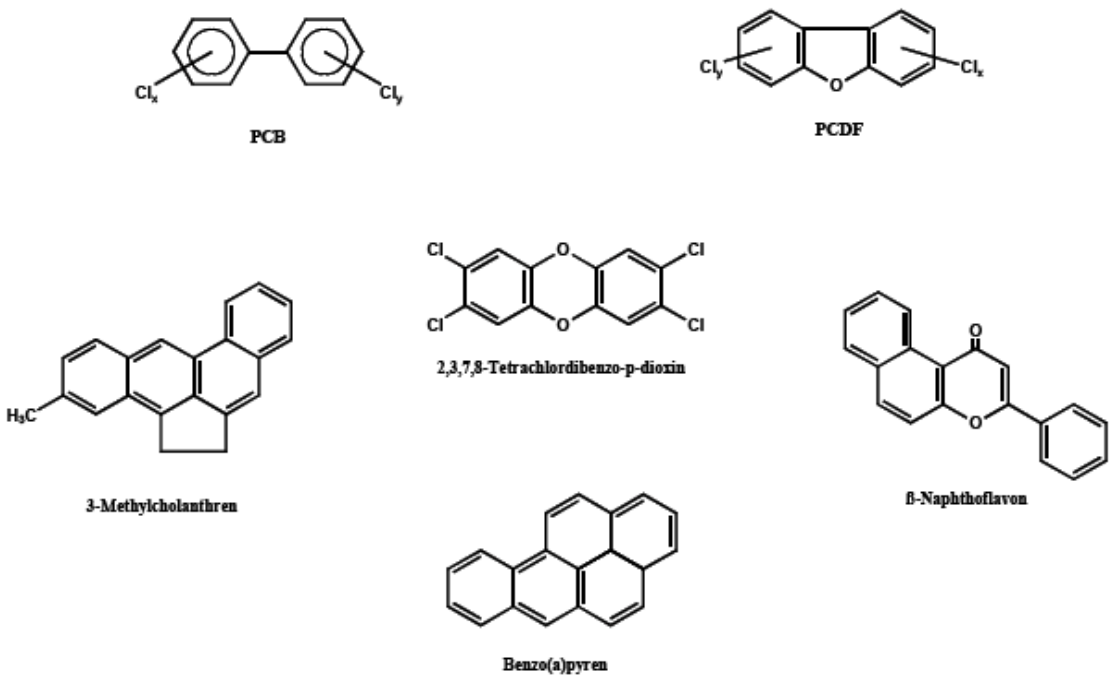


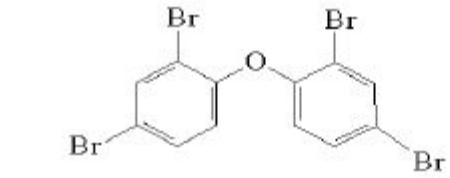
Abbildung 2: Strukturen von synthetischen AhR-Liganden

Die meisten PAK und HAK sind persistent in der Umwelt und entstehen bei Verbrennungsprozessen sowie bei der Synthese von Organochlorverbindungen als Verunreinigung. Dioxine und polyhalogenierte Biphenyle reichern sich aufgrund ihrer Hydrophobizität häufig in der Nahrungskette an und können in hohen Konzentrationen in fetthaltigen Lebensmitteln nachgewiesen werden, z.B. in Fleisch, Fisch und Milchprodukten und akkumulieren beim Menschen zum größten Teil im Fettgewebe mit einer Halbwertszeit von bis zu 10 Jahren (Pirkle et al. 1989). Die Hauptexpositionsquelle für andere PAK wie B[a]p und dem 3-MC stellt das Rauchen dar.

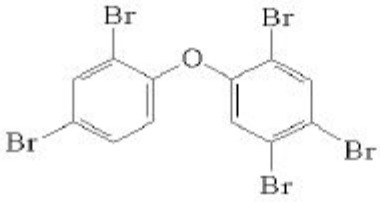
1.2.2 Polybromierte Diphenylether (PBDE)

Eine weitere Substanzklasse, die als potentielle synthetische AhR-Liganden diskutiert werden und deren Einfluss auf die neurale Entwicklung in Publikation 2.4 und 2.5 näher betrachtet wird, sind die polybromierten Diphenylether (PBDE). PBDE sind als Flammschutzmittel in vielen Verbrauchsgütern zu finden, z.B. in Computerbauteilen und Textilien. Ähnlich den PAKs und HAKs weisen sie auch eine hohe Bioakkumulation in der Umwelt auf und ihre Verwendung und Produktion in Europa und Nordamerika ist aufgrund dessen bereits stark eingeschränkt worden. Als besonders kritisch wird die Möglichkeit der Exposition gegenüber PBDE-Kongeneren von Säuglingen durch die Muttermilch betrachtet, da PBDE im Tierversuch neuronale Toxizität, Entwicklungstoxizität und endokrine Disruption

erzeugen (Darnerud 2003;Legler and Brouwer 2003). PBDE werden im Körper zum Teil metabolisiert. Experimentelle Studien von Meerts und Mitarbeitern (2000; 2001) belegten die Entstehung von hydroxylierten PBDE-Metaboliten nach Inkubation der PBDE-Kongenere in induzierten Leber-Mikrosomen und auch *in vivo* konnte die Existenz hydroxylierter PBDE-Metabolite in Fäzes und Urin von Ratten und Mäusen nachgewiesen werden (Hakk and Letcher 2003).



BDE47 (2,2´,4,4´-Tetrabromdiphenylether)



BDE99 (2,2´,4,4´,5-Pentabromdiphenylether)

Abbildung 3: Struktur der PBDE-Kongenere BDE47, BDE99. Die Nomenklatur der entsprechenden Verbindungen ist anhand einer festgelegten Nummerierung nach Anzahl und Position der Substitutenten durch die *International Union of Pure and Applied Chemistry* (IUPAC) bestimmt.

Die molekularen Mechanismen der ENT von PBDE sind bisher noch unklar, aber strukturelle Ähnlichkeiten der PBDE mit dioxin-ähnlich wirksamen Substanzen, z.B. mit polychlorierten Biphenylen (PCB) und bromierten Furanen und Dioxinen, lassen auf eine Beteiligung des AhR schließen (Abb. 3). Dies wird auch durch Studien unterstützt, die eine Modulation der AhR-Aktivität nach PBDE-Exposition nachweisen konnten (Chen and Bunce 2003;Hamers et al. 2006;Peters et al. 2006). Des Weiteren könnten Störungen des endokrinen Systems durch PBDE bei den beobachteten toxischen Effekten eine Rolle spielen. PBDE und verwandte Verbindungen haben bereits in *in vitro* Studien Potential gezeigt, mit dem Thyroid- und Steroidhormonsystem zu interferieren. Das PBDE-Kongener BDE47 bzw. sein

hydroxylierter Metabolit (6HO-BDE47) hatten eine stark anti-östrogene Wirkung (Hamers et al. 2006;Meerts et al. 2001). Auch antagonistische Wirkungen auf den Androgen- und den Progesteron-Rezeptor wurden in der Literatur beschrieben (Hamers et al. 2006). Weiterhin führte DE-71, eine kommerzielle PBDE-Mixtur, wie auch einzelne PBDE-Kongenere (z.B. BDE-47) zu einer Beeinträchtigung des Thyroid-Hormonsystems (Stoker et al. 2004) und auch für hydroxylierte PBDE-Metabolite ist beschrieben, dass sie mit dem Thyroidhormonsystem interferieren (Darnerud et al. 2001;Meerts et al. 2000).

Inwieweit PBDE direkt mit dem Thyroidhormonrezeptor wechselwirken können und welche Auswirkungen das auf die Entwicklung neuraler Progenitorzellen *in vitro* hat, wird in Publikation 2.4 ausführlich dargestellt. Dass auch eine Beeinflussung der Calcium-Homöostase eine Rolle bei der beschriebenen Entwicklungsneurotoxizität der PBDE spielen könnte, wird darauf folgend in Publikation 2.5 diskutiert.

1.2.3. Natürliche AhR-Liganden

Die größte Expositionsquelle von Organismen für natürliche AhR-Liganden ist die Nahrung. Die hier vorkommenden Liganden haben eine meist schwache Bindungsaffinität zum AhR. Hierzu gehören unter anderem Indol-3-carbinol (I3C), Carotinoide wie Canthaxanthin, Rutacarpine wie 7,8-Dihydrorutacarpin, Dibenzoylmethane und Flavonoide (Ashida et al. 2008;Moon et al. 2006). Diese Substanzklassen kommen hauptsächlich in pflanzlicher Nahrung oder Pflanzenextrakten vor, z.B. Gemüsen, Früchten, Kräutern oder Tee.
Flavonoide repräsentieren dabei die größte Gruppe an in Lebensmitteln vorkommenden AhR-Liganden. Hierzu gehören unter anderem Flavanole, Flavone, Flavanone und Isoflavone. Sie sind in einer Vielzahl von pflanzlichen Nahrungsmitteln, Extrakten und Pharmazeutika zu finden. Flavonoide fungieren als Agonisten und/oder Antagonisten des AhR und der AhR-vermittelten Genexpression insbesondere der CYP-Induktion. Einige Verbindungen haben sowohl agonistisches als auch antagonistisches Potential. Dazu gehören Quercetin, Galangin und Tangeretin, wobei Enzymeffekte und eine eventuelle antikanzerogene Wirkung sehr stark von der Konzentration der Substanzen sowie der experimentellen Methodik abhängig sind (Ashida et al. 2008;Canivenc-Lavier et al. 1996;Ciolino et al. 1998a;Ciolino et al. 1998b;Ciolino and Yeh 1999;Henry et al. 1999;Hodek et al. 2002;Jeuken et al. 2003;Lautraite et al. 2002;Shertzer et al. 1999;Siess et al. 1989;Zhai et al. 1998;Zhang et al. 2003).
Zu der Klasse der Flavonoide werden insgesamt ungefähr 6500 Vertreter gezählt, allesamt bestehend aus einem Grundgerüst aus zwei aromatischen Ringsytemen (Ring A und B),

verbunden über 3 Kohlenstoffatome, die einen O-heterozyklischen Ring bilden (Ring C) (Abb. 4). Die Vielfalt der Flavonoide lässt sich durch Modifizierungen an dem Grundgerüst, so z.B. Hydroxylierungen, Methylierung und Glykosidierung sowie diverse Konjugationen der aromatischen Ringe erklären. Eine Einteilung in die folgenden sechs Unterklassen erfolgt über strukturelle Unterschiede am heterozyklischen C-Ring: Flavonole, Flavanole, Flavanone, Flavone, Anthocyanidine und Isoflavone (Abb. 4).

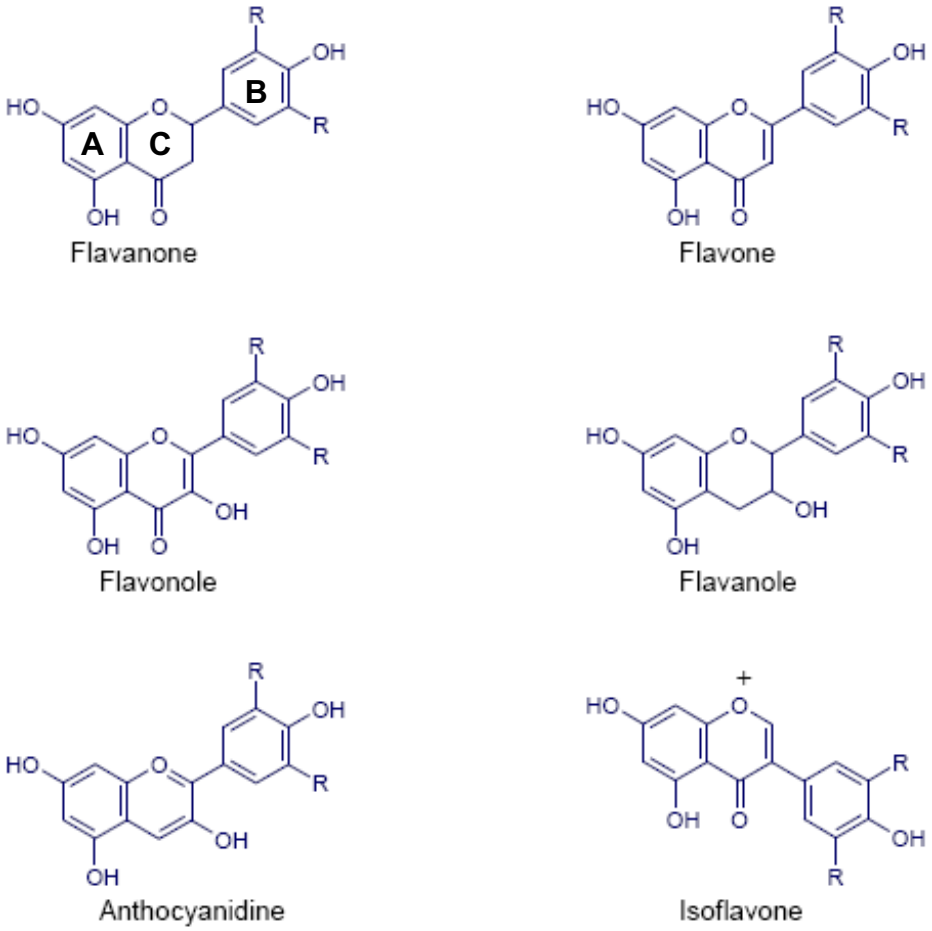


Abbildung 4: Strukturformeln der sechs Unterklassen der Flavonoide

In den letzten Jahren wurden viele Studien zur Wirkungsweise von Flavonoiden durchgeführt. In diesen Studien fand man unter anderem eine positive Korrelation zwischen der Aufnahme an Flavonoiden und zahlreichen positiven Auswirkungen auf die Gesundheit. So wurde z.B. eine Reduktion des Risikos für Atherosklerose und sukzessiv für Herzerkrankungen durch eine flavonoidreiche Ernährung beschrieben (Reed 2002). Darüber hinaus wurde ein Zusammenhang zwischen Flavonoidaufnahme und antikanzerogener Wirkung festgestellt (Kris-Etherton et al. 2002) und neuroprotektive sowie positive kognitive Auswirkungungen proklamiert (Youdim et al. 2002;Youdim and Joseph 2001). Auch das bekannte Phänomen des „french paradox“ wird den Polyphenolen zugeordnet (Renaud and de Lorgeril 1993).

Aufgrund dieser positiven Auswirkungen der Flavonoide auf die Gesundheit des Menschen werden sie vermehrt als Nahrungsergänzungsmittel angeboten. Diese beinhalten die entsprechenden Flavonoide in Reinform und eine kontrollierte Einnahme ist, auch wegen der rezeptfreien Verfügbarkeit, nicht gegeben. Auch sind die vom jeweiligen Hersteller empfohlenen Tagesdosen von zumeist 500 mg bis 1 g so hoch, dass deutlich höhere Konzentrationen im Plasma oder in den Zellen entstehen können als durch eine natürliche Aufnahme der Flavonoide mit der Nahrung. Zudem zeigen neuere Erkenntnisse, dass viele Wirkungen der Flavonoide nicht den klassischen antioxidativen Eigenschaften zuzuschreiben sind, sondern über eine Beeinflussung diverser zentraler Signalkaskaden wirken (Williams et al. 2004). Gerade die positiven Wirkungen von Flavonoiden auf kognitive Fähigkeiten sowie auf alterungsbedingte Veränderungen im Gehirn unterstützen die Theorie, dass Flavonoide Signalmolekülcharakter tragen (Casadesus et al. 2004;Joseph et al. 2005), denn die Blut-Hirn-Schranke stellt eine Barriere mit geringer Permeabilität für polare Stoffe wie Flavonoide dar, was eine äußerst geringe Konzentration solcher Substanzen im Gehirn nach sich zieht. Die Konzentrationen anderer kleiner antioxidativer Moleküle im Gehirn, wie z.B. Ascorbinsäure oder α-Tocopherol, sind vielfach höher und sollten daher in der gesamten antioxidativen Kapazität eine wesentlich wichtigere Rolle spielen als die Flavonoide (Spencer 2008). Dass Flavonoide tatsächlich in zelluläre Signalwege eingreifen können, ist in vielen *in vitro*- und *in vivo*-Studien gezeigt worden. So können Flavonoide wie bereits erwähnt den AhR-Signalweg modulieren. Aber auch Interaktionen mit Proteinkinase-Signalwegen, wie z.B. dem Phosphoinositol-3-Kinase (PI3K)/Akt-Signalweg, dem MAP (Mitogen aktivierte Protein) Kinase-Signalweg, oder der Protein Kinase C (PKC) und weiteren Tyrosinkinasen (Agullo et al. 1997;Fresco et al. 2006;Teillet et al. 2008) sind bereits in der Literatur beschrieben. Inwieweit Flavonoide in die humane neurale Entwicklung *in vitro* eingreifen, kann anhand der Publikation 6 nachverfolgt werden, die sich mit dem Einfluss ausgewählte Flavonoide auf die Migration und Adhäsion von humanen neuralen Progenitorzellen beschäftigt.

1.2.4 Endogene AhR-Liganden

Auch in der Abwesenheit von exogenen Liganden ist der AhR-Signaltransduktionsweg aktiviert, was für die Existenz von endogenen Liganden des AhR spricht (Nguyen and Bradfield 2008). Es wurde bereits nachgewiesen, dass Tryptophanderivate und Indol(3,2-b)carbachol (Kleman et al. 1992;Rannug et al. 1995), Bilirubin (Sinal and Bend 1997) und Arachidonsäuremetabolite (Denison and Nagy 2003;Schaldach et al. 1999) mit hoher Aktivität an den AhR binden. Auch die Indigoide Indirubin und Indigo sind äußerst potente

Induktoren des AhR, die im menschlichen Urin gefunden wurden. Die Induktion des AhR durch Indigo, Indirubin und Indirubin-3´-oxim ist transient und abhängig von der CYP1A1 Induktion, was auf eine selbstaktivierte Metabolisierung der Substanzen zurückzuführen ist. Ob diese Indigoide wirklich zu den endogen gebildeten Liganden gehören, muss noch geklärt werden (Adachi et al. 2001;Denison and Nagy 2003;Eisenbrand et al. 2004;Hoessel et al. 1999;Marko et al. 2001;Miller, III 1997;Spink et al. 2003;Sugihara et al. 2004).

1.3 Neurale Progenitorzellen als *in vitro* Testsystem für Entwicklungsneurotoxizität

1.3.1. Gehirnentwicklung des Menschen

Das sich entwickelnde Zentralnervensystem des Menschen ist durch komplexe biologische Prozesse gekennzeichnet, welche über den langen Zeitraum der neuralen Entwicklung bis hin zur Pubertät strengen Kontrollmechanismen unterliegen. Dabei wird eine hohe Anzahl an verschiedenen Zelltypen gebildet, die unterschiedlichste Aufgaben während der Entwicklung des Gehirns übernehmen. Die Komplexität der biologischen Prozesse sowie deren zeitliche Staffelung sind in Abbildung 5 dargestellt.

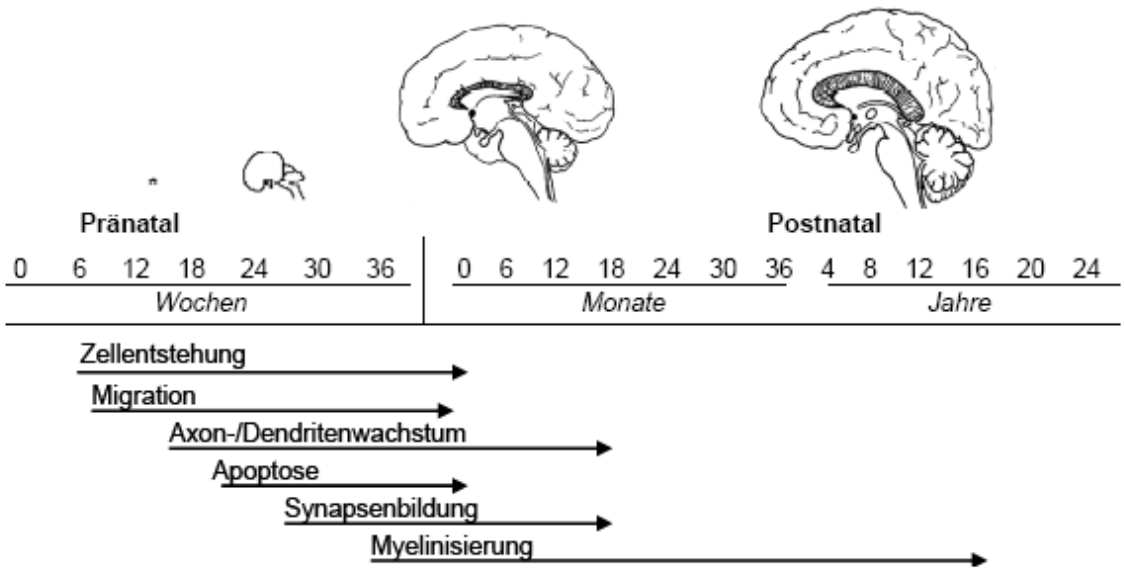


Abbildung 5: Zeitlicher Phasenverlauf der Gehirnentwicklung des Menschen (nach Andersen 2003)

Während der Hirnentwicklung müssen Proliferation von Progenitorzellen, Migration, Differenzierung, Verknüpfung mit Synapsen- und Netzwerkbildung, Myelinisierung sowie Apoptose zeitlich koordiniert erfolgen, um die äußerst komplexe Funktion dieses Organs zu gewährleisten. Hierzu gehört z.B. die Kontrolle des Zellzyklus, besonders die symmetrischen und asymmetrischen Zellteilungen der neuralen Epithelzellen zum Erhalt und zur Proliferation von Stammzellpopulationen und Progenitorzellen. Ebenso werden zellzahlregulierende Prozesse, z.B. die Apoptose bestimmter Zellpopulationen, zu gewissen Zeiten und in bestimmten Regionen durch Signalkaskaden und extrazelluläre Stimuli exakt reguliert. Die Determinierung der Progenitorzellen in unterschiedliche Zellpopulationen zu differenzieren, stellt einen wichtigen Regulierungsschritt in der Neurogenese dar. Auch die Zellmigration der Progenitorzellen aus der ventrikularen bzw. subventrikularen Zone zu den Endpositionen ist ein streng kontrollierter Prozess.
Diese große Anzahl an möglichen Interaktionspunkten mit den verschiedenen Prozessen der Hirnentwicklung macht das entstehende Gehirn sensibel gegenüber exogenen Noxen. Es gibt kritische Perioden der Sensitivität, da einzelne Prozesse zu konkreten Zeitpunkten und somit in spezifischen Entwicklungsstadien auftreten (Andersen 2003;Rice and Barone S Jr 2000). Ebenso ist das sich entwickelnde Gehirn durch eine noch nicht vollständig ausgebildete Blut-Hirn-Schranke nicht so effektiv geschützt wie ein postnatales Gehirn, so dass sogar Ionen und hydrophile Noxen in das fetale Gehirn gelangen können (Claudio et al. 2000). Auch ist das fremdstoffmetabolisierende Enzymsystem im Embryo/Fetus erst sehr rudimentär ausgeprägt (Bondy and Campbell 2005).

1.3.2 Entwicklungsneurotoxizität (ENT)

Im Rahmen entwicklungsneurotoxikologischer Studien werden die Effekte exogener Substanzen auf das sich entwickelnde Nervensystem untersucht. Die Exposition des sich entwickelnden Gehirns kann dabei durch die Mutter während der Schwangerschaft oder durch das neugeborene Kind selbst erfolgen und pathologisch relevante Veränderungen bewirken (Andersen et al. 2000). Solche Fremdstoffexpositionen können über ein Eingreifen in die zuvor beschriebenen zelluläre Strukturen oder Signalwege die Leistungsfähigkeit des sich entwickelnden Gehirns verringern und neurologische Defizite hervorrufen. Zu den Substanzen, die beim Menschen als entwicklungsneurotoxisch beschrieben sind, gehören Quecksilber, Blei, Arsen, Polychlorierte Biphenyle und Toluol (Grandjean and Landrigan 2006). Kinder, welche gegenüber diesen Stoffen exponiert waren, zeigten u.a. Lern- und Gedächtnisstörungen. Von vielen sich im Umlauf befindlichen Chemikalien ist ihr entwicklungsneurotoxisches Potential nicht bekannt. Es weisen jedoch mehr als 10 % der

Kinder neurologische Defizite auf, welche möglicherweise neben genetischer Prädisposition und sozialen Umständen auch auf eine Exposition gegenüber entwicklungsneurotoxischen Substanzen zurückgeführt werden können (Schettler 2001).

1.3.3. Entwicklungsneurotoxikologische Testsyteme

Im Bereich der ENT sind zurzeit noch Tierversuchstudien zur Risikoabschätzung von Chemikalien vorgeschrieben. Es stehen in diesem Zusammenhang eine Richtlinie der U.S. EPA und die OECD Richtlinie 426 zur Verfügung. In diesen Richtlinien werden morphologische Gehirnuntersuchungen der Versuchstiere, Verhaltenstests und die Untersuchung von Biomarkergenen für Gliosen und Zytotoxizität aus *in vivo* Studien gefordert. Solche Tierversuchsstudien sind jedoch sehr kostenaufwendig und zeitintensiv. Allein die Anzahl benötigter Tiere liegt bei etwa 140 Muttertieren und 1000 Jungtieren, was die Durchführung von Tierversuchsstudien für die Chemikalientestung und Registrierung von mehreren tausend Chemikalien im Rahmen von REACH unmöglich macht. Deshalb gewinnt die Entwicklung und Validierung von Alternativmodellen, die ein Screenen der zu testenden Chemikalien ermöglichen, zunehmend an Bedeutung.

Als zum Nagetier alternative *in vivo* Modelle zur Untersuchung von ENT stehen Vertebraten wie der Zebrafisch, der Medakafisch und der wirbellose Modellorganismus *Caenorhabditis elegans* im Vordergrund. Diese Alternativmodelle ermöglichen die Untersuchung toxischer Effekte *in vivo* und erhalten somit die Komplexität der Physiologie des Gesamtorganismus. Gegenüber Nagetierstudien weisen diese Modelle diverse Vorteile auf. Sie haben eine geringe Größe, eine kurze Generations-, Embryonal- und Lebenszeit. Diese Aspekte ermöglichen schnell durchzuführende Generationsstudien und eine Kultivierung der Organismen in 96-well-Platten, so dass auch Hoch-Durchsatzuntersuchungen möglich sind. Zudem bietet die morphologische Transparenz der Embryos die Möglichkeit, Veränderungen in der Entwicklung neuraler Organe leicht und routinemäßig zu untersuchen (Coecke et al. 2007).

Grundlegende Prozesse der Gehirnentwicklung können zu einem gewissen Teil in *in vitro* Systemen nachgestellt werden. So können Prozesse wie Proliferation, neurale Differenzierung, neuronale Reifung und Apoptose in verschiedenen Zellsystemen beobachtet werden. Als potentielle *in vitro* Modelle stehen Stammzellen, immortalisierte Zelllinien und auch Gehirnaggregate verschiedener Spezies zur Diskussion. Auch primäre Zell- und Organkulturen scheinen vielversprechend zu sein, wobei Organkulturen den Vorteil aufweisen, dass die dreidimensionale *in vivo* Struktur bestehen bleibt (Coecke et al. 2007). Während sich fast alle Modellsysteme eignen, Einflüsse auf die Zellproliferation (mit Ausnahme von Organkulturen) und die Apoptose zu untersuchen, treten bei der Bestimmung

der weiteren Endpunkte Differenzen zwischen den unterschiedlichen Modellsystemen auf. So sind Stammzellen sehr gut für eine Untersuchung der Zelldifferenzierung geeignet, während die Prozesse der Myelinisierung und der neuronalen Reifung eher in primären Kulturen und Organschnitten zu untersuchen sind. Eine Bewertung der immortalisierten Zelllinien zeigt zwar, dass auch in diesen Modellsystemen die diskutierten Endpunkte mit Ausnahme der neuronalen Reifung etabliert sind. Da es sich jedoch um entartete Zellen handelt, kann nicht von einer direkten Übertragbarkeit der Daten auf normale Zellen ausgegangen werden.

Zur Vertiefung dieser Thematik sei auf Publikation 2.1 im Hauptteil der Dissertation verwiesen, die eine ausführliche Beschreibung von neuralen Stamm-/Progenitorzellen als Modelle in der Entwicklungsneurotoxikologie beinhaltet.

1.3.4 Neurosphären bilden basale Prozesse der Gehirnentwicklung *in vitro* ab

In der vorliegenden Dissertation wurden zur ENT-Testung normale neurale Progenitorzellen (NPCs) humanen und murinen Ursprungs verwendet, die als Neurosphären kultiviert wurden. Progenitorzellen, auch Vorläuferzellen oder determinierte Stammzellen genannt, sind Tochterzellen einer pluripotenten Stammzelle adulten oder embryonalen Ursprungs, welche bereits auf ein Zielorgan determiniert sind, aber trotzdem auf die Regenerationseigenschaften bezogen Stammzellcharakter behalten. NPCs können beispielsweise aus Vollhirnhomogenaten menschlicher oder muriner Feten gewonnen werden. In Suspensionskultur werden die humanen NPCs vermehrt und bilden in Gegenwart von Wachstumfaktoren dreidimensionale Zellaggregate, welche Neurosphären genannt werden (Buc-Caron 1995;Chalmers-Redman et al. 1997;Reynolds et al. 1992;Svendsen et al. 1995). Neurosphären können zudem aus einer einzigen neuralen Stammzelle generiert werden. Klonale Analysen zeigten aber, dass nur etwa 2,25% der Zellen einer Neurosphäre diese proliferativen Eigenschaften besitzen (Reynolds and Rietze 2005). Die Neurosphären werden bis zu drei Monate in Suspensionskultur gehalten und können aufgrund der proliferativen Größenzunahme durch mechanische Zerkleinerung passagiert werden (Svendsen et al. 1998). Sie repräsentieren eine heterogene Zellpopulation, die Marker neuraler Stammzellen und früher Progenitorzellen exprimieren (Brannen and Sugaya 2000;Messina et al. 2003;Piper et al. 2000;Piper et al. 2001). In mehreren Studien konnte gezeigt werden, dass neurale Progenitorzellen aus Neurosphären *in vitro* und *in vivo* in Glia- und neuronale Zellpopulationen differenzieren, die zudem die Fähigkeit aufweisen, *in vivo* zu migrieren (Brannen and Sugaya 2000;Flanagan et al. 2006;Maciaczyk et al. 2009;Mothe et

al. 2008;Piao et al. 2006;Piper et al. 2001;Svendsen et al. 1997). Die Grundzüge der Neurosphärenkultur mit ihren wichtigen beiden Grundeigenschaften, der Regeneration und der Multiplizität, sind in Abbildung 6 noch einmal zusammengefasst.

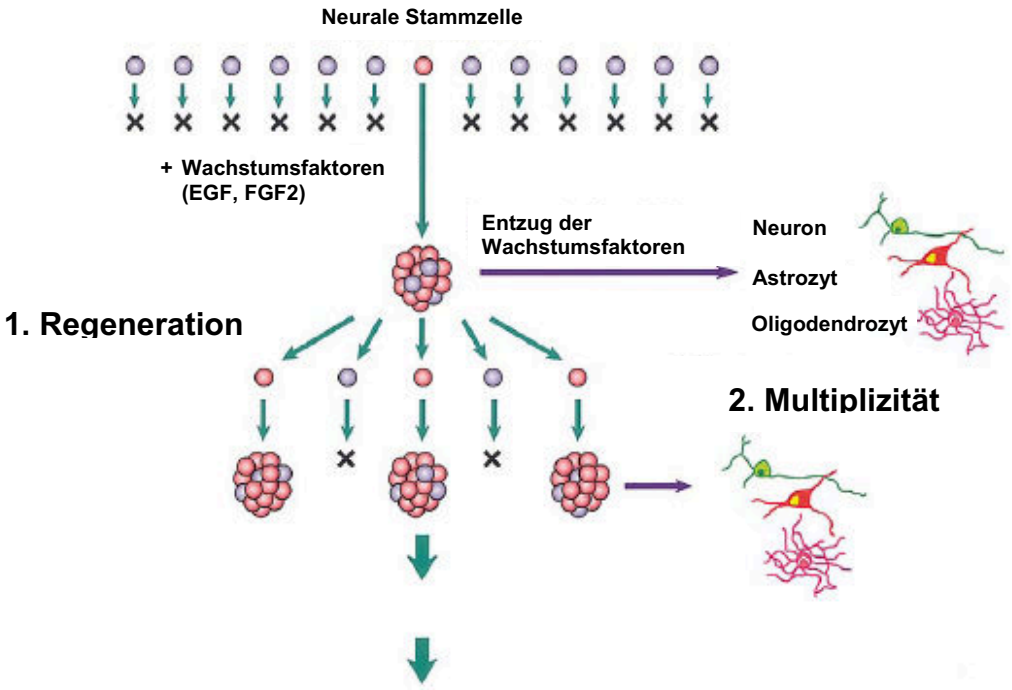


Abbildung 6: Das Neurosphärenmodellsystem modifiziert nach Okano (2002).

Ein Vergleich von fetalen Gehirnen und Neurosphären ergab zusätzlich, dass Stamm- und Progenitorzellen aus Neurosphären entsprechenden *in vivo* Korrelaten hinsichtlich der proliferativen Kapazität und der Modulation der Differenzierung nach Zugabe exogener Noxen ähneln. Zusätzlich bleibt die spezifische Expression vieler entwicklungsrelevanter Gene in Neurosphären erhalten (Jensen and Parmar 2006), was die Nähe zu physiologischen Bedingungen verdeutlicht.

Der klare Vorteil dieser Kulturform gegenüber der konventionellern „Monolayer-Kultur“ ist, dass sich die Zellen in der Sphäre selbst organisieren. Auch in dem sich entwickelnden Gehirn sind verschiedene Reifungsgrade der Zellpopulationen in unmittelbarer Nähe zu finden (Andersen 2003). Daher wird postuliert, dass Neurosphären einzelne Aspekte der *in vivo* Situation *in vitro* abbilden. Zudem differenzieren und migrieren transplantierte humane Vorläuferzellen aus Neurosphären *in vivo* im Mäusegehirn, was ebenfalls für die Physiologie der Neurosphäre spricht (Reubinoff et al. 2001).

Die Forschung mit Neurosphären begründet sich zumeist auf der Anwendung als Therapie für neurodegenerative Erkrankungen oder in der Regenerationsmedizin (Burnstein et al. 2004;Ebert et al. 2008;Kelly et al. 2004). Die Arbeiten unserer AG haben jedoch gezeigt,

dass sich dieses Modell sehr gut zur toxikologischen Untersuchung verschiedener Stoffe auf die basalen Prozesse der Gehirnentwicklung, z.B. Proliferation, Migration und Differenzierung eignet (Fritsche et al. 2005;Moors et al. 2007;Moors et al. 2009).

1.4 Ziel der Dissertation

Folgende Aufgabenstellungen wurden in der vorliegenden Dissertation bearbeitet:

(i) Vertiefende molekulare Charakterisierung der humanen neuralen Progenitorzellen und Etablierung/Optimierung von Assays für verschiedene Endpunkte, um das Potential des Neurosphären Modellsystems zur Neurotoxizitätstestung bewerten zu können.

(ii) Untersuchung der Rolle des Arylhydrokarbonrezeptors in der humanen neuralen Entwicklung durch Exposition der hNPCs mit unterschiedlichen synthetischen und natürlichen potentiellen AhR-Liganden zur Aufklärung von molekularen Mechanismen und als Grundlage für eine Risikobewertung der entsprechenden Verbindungen.

(iii) Interspezies-Vergleich der AhR-vermittelten Wirkungen anhand von murinen NPCs.

2. Manuskripte

Im Folgenden sind die Publikationen als Erst- und Co-Autor angefügt. Bei der ersten Publikation (2.1) handelt es sich um einen Review mit dem Titel: „Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: State of the science". In dieser Publikation werden die aktuell zur Verfügung stehenden auf neuralen Progenitorzellen beruhenden *in vitro* Modellsysteme beschrieben und ihre Einsetzbarkeit für neuroentwicklungstoxikologische Studien im Hochdurchsatzverfahren diskutiert. Nach dieser generellen Einführung in die Thematik wird in der darauf folgenden Publikation „Human Neurospheres as Three-Dimensional Cellular Systems for Developmental Neurotoxicity Testing" das für diese Arbeit verwendete auf normalen humanen neuralen Progenitorzellen beruhende Neurosphären-Testsystem vorgestellt (2.2). Dieses *in vitro* Modellsystem ist geeignet, die basalen Prozesse der Gehirnentwicklung Migration, Differenzierung, Proliferation und Apoptose abzubilden und entwicklungsneurotoxische Noxen zu erkennen. Auf dieser Grundlage konnten in den folgenden Publikationen die Effekte von unterschiedlichen potentiellen synthetischen und natürlichen AhR-Liganden aufgeklärt werden. In Publikation 2.3 wird zunächst die Wirkung klassischer synthetischer AhR-Liganden untersucht. Im Vordergrund steht dabei der direkte Vergleich zwischen humanen und murinen Neurosphären. Die beiden darauf folgenden Publikationen (2.4 & 2.5) beschäftigen sich mit dem Einfluss von PBDEs auf die menschliche Gehirnentwicklung *in vitro*. Da in der Veröffentlichung 2.3 herausgefunden wurde, dass der AhR in humanen Neurosphären nicht exprimiert wird, fokussieren diese Arbeiten auf eine Interferenz von PBDE mit der Thyroidhormon- und der Calcium-Signaltransduktion. Die letzte Publikation (2.6) beschreibt dann die Effekte von ausgesuchten Flavonoiden besonders auf die Endpunkte Zellmigration und –adhäsion mit Schwerpunkt auf der Integrin-Laminin-Interaktion.

2.1 Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: State of the science

Breier JM, **Gassmann K**, Kayser R, Stegeman H, De Groot D, Fritsche E, Shafer TJ.
Neurotoxicol Teratol. [zur Publikation angenommen am 24. Juni 2009]

Es sind schätzungsweise 100.000 Altstoffe im Umlauf, die keiner toxikologischen Testung unterzogen wurden. Im REACH Programm der Europäischen Union sollen von diesen ca. 30.000 Substanzen mit einer jährlichen Produktion von über einer Tonne hinsichtlich ihres toxischen Potentials bewertet werden, wobei entwicklungsneurotoxische Untersuchungen für Substanzen mit teratogenen und neurotoxischen Eigenschaften empfohlen werden. Bis heute existiert neben zeit- und kostenaufwendigen Tierversuchen jedoch kein geeignetes validiertes Modellsystem zur Durchführung solcher Studien. Demnach ist die Etablierung von alternativen *in vitro* Testmethoden zur Bewertung des entwicklungsneurotoxischen Potentials diverser Chemikalien dringend notwendig. Die hohe Anzahl der zu testenden Chemikalien macht es darüber hinaus erforderlich, dass die entsprechenden *in vitro* Testmethoden sich für eine Durchführung im Hochdurchsatzverfahren eignen.

Besonders wichtig bei der Wahl des geeigneten Testsystems ist, dass es die humane Gehirnentwicklung möglichst wirklichkeitsgetreu abbildet und dabei einen hohen Grad an Sensitivität und Spezifität aufweist. Bis vor kurzem beschränkten sich die verfügbaren Modellsysteme hauptsächlich auf transformierte Zelllinien und primäre Nagerzellmodelle. Das Problem bei rein murinen Testsystemen ist, dass die Übertragbarkeit auf den Menschen nicht immer gewährleistet ist. Bei primären neuralen Kulturen kommt zusätzlich noch eine geringe Überlebensdauer in Kultur hinzu. Krebszelllinien humanen Ursprungs umgehen diese beiden Probleme, aber weisen den Nachteil auf, dass sie aufgrund ihrer Transformation keiner normalen physiologischen Regulation mehr unterliegen.

Aus diesen Gründen sind in den letzten Jahren verstärkt neurale Progenitorzellen humanen und murinen Ursprungs in den Mittelpunkt des Interesses gerückt. NPC weisen das Potential der Selbsterneuerung und einer fast unbeschränkten Lebenszeit auf und unter geeigneten Bedingungen ist eine Differenzierung in alle drei dominanten Zellpopulationen des Gehirns, Astrozyten, Oligodendrozyten und Neurone möglich. Sie können aus verschiedenen Arealen des fetalen und adulten Gehirns und auch aus Nabelschnurblut isoliert werden und sind mittlerweile auch kommerziell verfügbar. NPC können in Form von adhärenten Monolayern oder auch in dreidimensionalen Zellaggregaten, sogenannten Neurosphären, kultiviert werden.

Die Anzahl publizierter Daten im Bezug auf entwicklungsneurotoxikologische Endpunkte in NPC ist bisher noch gering und beschränkt sich in den meisten Fällen auf die Exposition mit endokrinen Disruptoren, Ethanol, Methylquecksilberchlorid und Blei. Für die Validierung

eines auf NPC beruhenden alternativen *in vitro* Testsystems für ENT fehlen bisher noch Studien mit einer größeren Anzahl von systematisch ausgewählten Testchemikalien und mehr Daten zur allgemeinen Zellbiologie der NPC. Die bisherigen Ergebnisse zeigen jedoch deutlich, dass neurale Progenitorzellen das momentan geeigneteste Zellmodell sind, um die menschliche Gehirnentwicklung *in vitro* wiederzuspiegeln. Zudem ähneln ihre zellulären Reaktionen auf exogene Noxen den Zellantworten, die *in vivo* beobachtet wurden. Erste Untersuchungen weisen darüber hinaus darauf hin, dass zumindest ein mittlerer Probendurchsatz mit NPCs realisierbar ist.

Die Verfasserin der Dissertation war maßgeblich an der Erstellung der Kapitel, die sich mit der Beschreibung des Neurosphärenmodells und seiner Nutzung in der toxikologischen Testung beschäftigen, beteiligt.

Neurotoxicology and Teratology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: State of the science ☆

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ARTICLE INFO

Article history:
Received 23 January 2009
Received in revised form 1 June 2009
Accepted 8 June 2009
Available online xxxx

Keywords:
Stem cell
Neuroprogenitor cells
Developmental neurotoxicity screening
Neurosphere
Cell culture
High-throughput screening

ABSTRACT

In vitro, high-throughput methods have been widely recommended as an approach to screen chemicals for the potential to cause developmental neurotoxicity and prioritize them for additional testing. The choice of cellular models for such an approach will have important ramifications for the accuracy, predictivity and sensitivity of the screening assays. In recent years neuroprogenitor cells from rodents and humans have become more widely available and may offer useful models having advantages over primary neuronal cultures and/or transformed cell lines. To date, these models have been utilized in only a limited number of toxicity studies. This review summarizes the state of the science regarding stem and neuroprogenitor models that could be used for screening assays, provides researchers in this field with examples of how these cells have been utilized to date, and discusses the advantages, limitations and knowledge gaps regarding these models. Data are available from both rodent and human stem and neuroprogenitor cell models that indicate that these models will be a valid and useful tool for developmental neurotoxicity testing. Full potential of these models will only be achieved following advances in neurobiology that elucidate differentiation pathways more clearly, and following further evaluation of larger sets of developmentally neurotoxic and non-toxic chemicals to define the sensitivity and predictivity of assays based on stem or progenitor cell models.

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1. Introduction

The first in vitro culture of neural tissue was developed slightly over 100 years ago for the purpose of studying neurite outgrowth [45]. Since then, cell culture techniques have undergone numerous refinements and have become a proven approach for understanding the development, structure and function of the nervous system (for review, see [116]). In the field of neurotoxicology, cell culture has been used primarily for studies of mechanism of chemical action, and to a much lesser extent for toxicity screening. One challenge has always been to derive in vitro models that accurately recapitulate in vivo nervous system development. This is important to neurotoxicology, since conclusions regarding the mechanisms of chemical action on the developing nervous system are only as valid as the model system from which they are derived. Ultimately, for developmental neurotoxicology, predicting potential risks of chemical exposure in humans requires having appropriate models of the processes critical for neurodevelopment.

Until recently, the in vitro model systems that were available to study nervous system development and chemical effects thereon were limited to transformed cell lines from humans (e.g. SH-SY5Y) or rodents (PC12), primary cultures of rodent CNS, or tissue from aborted fetuses or resectioned from brain surgery patients. While these models serve a useful purpose, they also have some significant drawbacks as models of the developing nervous system. Transformed cell lines, whether of human or rodent origin, are typically derived from tumors, and likely do not represent the true state of native neural cells. In some instances, for example PC12 cells that have been widely used in developmental neurobiology and toxicology, the cell lines are not derived from neural tissue. By contrast, primary cells are not transformed, but in many cases these cultures contain populations of post-mitotic neurons and thus are less useful for studying early developmental processes such as

☆ Preparation of this document has been funded in part by the U.S. Environmental Protection Agency. This document has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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0892-0362/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.ntt.2009.06.005

progenitor proliferation and fate determination. In addition, since these cells are not self-renewing, new cultures must be prepared on a regular basis from freshly dissected animal tissue. Tissue from fetuses (from elective and spontaneous abortions) can be difficult to obtain on a regular basis in large quantities. Resectioned nervous system tissue obtained from surgery patients may contain non-normal cells, for example epileptic foci [116]. While the model systems described above have proved invaluable in understanding nervous system mechanisms, their limitations make them less than ideal models of nervous system development.

Recently, there has been increasing awareness and concern regarding the large number of chemicals for which toxicity data are incomplete and/or lacking, including the potential for developmental neurotoxicology [43,113]. One proposed solution to this problem is to utilize high-content/high-throughput screening (HCS/HTS) approaches to identify chemicals that may disrupt nervous system development and to prioritize them for further testing. The vast majority of available HCS/HTS approaches rely on in vitro models and tissue. In order to provide the highest level of predictivity for developmental neurotoxicity, it will be important that the models employed are accurate recapitulations of processes important for nervous system development, such as proliferation, migration, differentiation, and synaptogenesis. For this purpose, several reviews and symposia summaries have proposed the use of embryonic stem cells (ESCs) or neuroprogenitor cells (NPCs), including those of human origin [22,63,64]. Indeed, ESCs or NPCs may offer distinct advantages over other in vitro model systems. They offer the promise of a renewable source of normal cells with the capability to differentiate into any cell present in the nervous system. With respect to neurotoxicity, human embryonic stem (hESC) and neuroprogenitor cells (hNPC) offer the advantage of better predictive power since extrapolation of results across species is not at issue. However, a review of the state of the science related to the use of stem cells in drug and chemical screening has not been available to date. Therefore, the goal of this article is to review current knowledge regarding rodent and human stem and neuroprogenitor cell models as they could be applied to HCS/HTS, identify the strengths and limitations of those models, and point out areas in which additional information is needed in order to improve the utility of the models or improve and/or better understand their predictive capabilities.

2. Characteristics of stem and neuroprogenitor cells as related to HCS/HTS

Research in stem cell biology over the past decade has focused largely on the use of stem cells in transplantation therapy for various disease models. However, these cells have the potential to become powerful tools in developmental neurobiology and toxicology. Embryonic Stem Cells (ESCs) are isolated from the inner cell mass of blastocyst stage embryos. By definition, true stem cells are pluripotent, retaining the ability to differentiate into the three major germ layers of the embryo—endodermal, ectodermal and mesodermal—and theoretically into any cell type in the body. Multipotent neuroprogenitor cells (NPCs) are derived from cells of ectodermal lineage and are restricted in fate to develop into neurons, astrocytes and oligodendrocytes. The adult nervous system also contains populations of multipotent progenitor cells that can give rise to neurons, astrocytes and oligodendrocytes. Typically, stem cells, neuroprogenitor cells and differentiated cells are characterized by the expression of mRNA for, or immunoreactivity to, one or more phenotypic markers (Table 1).

For the purpose of screening thousands of potential developmental neurotoxicants and prioritizing them for additional testing, an in vitro model should have a number of basic characteristics. First, the cells must be easy to expand and replenish, in order to be able to generate the volumes of cells that would be needed to conduct HCS/HTS assays in multi-well plates. This demand for large volumes of cells creates a potential problem as, over time, cells in culture may lose genotypic and/or phenotypic stability or become contaminated with other cell types. This problem has been highlighted recently [51] as scientifically and financially costly. In the United States, the National Institute of Health has issued a formal statement urging all users of cell lines to authenticate the characteristics of the cells that they are using [10]. A second important characteristic is that the cells be easy to obtain. It is impractical to think that many of those interested in screening chemicals for the potential to cause developmental neurotoxicity will also have the resources to isolate and characterize stem and/or progenitor cells on a routine basis. Thus, for most neurotoxicologists, stable, self-replicating ESC or NPC models will have to be obtained either by sharing or gifting from others, from commercial sources, or from cell banks. Another characteristic that is needed for the purpose of screening is that the basic neurodevelopmental processes like proliferation, migration and differentiation be well characterized and reproducible. The conditions required for cells to differentiate, for example, the growth factors to be added to or withdrawn from the medium, the type of substrate (poly lysine, laminin, etc.) needed, and the amount of time required, need to be well understood and reproducible over time and across laboratories. In addition, the endpoints that characterize a differentiated population need to be well defined and reproducible. For any given NPC line, differentiation into neurons, astrocytes and oligodendrocytes should yield similar percentages of each cell type over time such that toxicant-induced changes in the proportions of each, or in the time-course can be reliably detected. Because many HCS/HTS approaches rely on imaging of biochemical and/or morphological endpoints in individual cells (neurite outgrowth, neurite number and average length, cell size and shape, nucleus/cytoplasm ratio), the ability to culture the cells on a monolayer may be preferable for many assays. However, as discussed below, some functional assays like cell migration may work well in three dimensional cultures such as neurospheres.

Table 1
Typical markers to identify pluripotent stem, multipotent neuroprogenitor, and differentiated neural cells.

Cell type	Markers
Stem cell (pluripotent)	NANOG, OCT4, SOX2, (rodent and human) TDGF1 DNMT3B GABRB3, GDF3 (hESC)
Neuroprogenitor (multipotent)	Nestin, SOX2, SSEA-1,
Neurons	βIII-tubulin, MAP2a, 2b
Glia	GFAP, S-100 protein
Oligodendrocytes	O4, O1

Abbreviations: TDGF1, teratocarcinoma-derived growth factor1; OCT4, octamer 4; DNMT3B, DNA methyltransferase 3 beta; GABRB3, gamma amino-butyric acid receptor-a beta 3 subunit; GDF3, growth differentiation factor 3; SOX2, SRY (sex determining region Y)-box 2; SSEA-1, stage-specific embryonic antigen 1; MAP2, microtubule associated protein; GFAP, glial fibrillary acidic protein; O1, oligodendrocyte marker 1; O4, oligodendrocyte marker 4.

3. Currently available models of stem and neuroprogenitor cells

There are a wide variety of different types of stem or neuroprogenitor cell models described in the published literature, and a comprehensive review of all of those models is beyond the scope of this paper. The following sections will discuss several of those models that have been used in or could be used for testing the effects of chemicals on neural tissue, and will describe rodent and human models as well as neurospheres, which are a special culture form of NPC.

3.1. Rodent models

Rodent ESC and NPC have been derived from mice or rats, and their embryos. Compared to human models, animal models offer several advantages including greater ease in deriving cells from different

Please cite this article as: J.M. Breier, et al., Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: State of the science, Neurotoxicol. Teratol. (2009), doi:10.1016/j.ntt.2009.06.005

developmental stages and brain structures, greater availability of fetal tissue, and greater consistency between tissues/cells from different individuals. Animal models also avoid the ethical concerns of working with human embryos.

Several rodent cell models are now commercially available. These include NPCs isolated from the hippocampus of adult rats as well as from embryonic mouse cortex and spinal cord (Millipore, USA). These cells typically grow in monolayers, have been characterized by the vendor to express marker proteins characteristic of NPCs, and are stable for several passages. Recently, mouse cortical and hippocampal cells that constitutively express green fluorescent protein have also been made commercially available. To date, we are unaware of any toxicity studies in the peer-reviewed literature that have utilized any of these models.

One available cell model derived from mice includes ES-D3 embryonic stem cells. These cells were first isolated from the blastocyst of the 129/Sv mouse [33]. Undifferentiated ES-D3 cells proliferate on non-adherent culture dishes and form cell clusters called embryonic bodies (EBs). Transfer of these EBs to adhesive tissue culture treated flasks and the use of serum-free insulin/transferrin/selenium (ITS) culture medium containing fibronectin induces migration of single cells out of the EBs. Finally, differentiation of these single cells into neural cell types [60] starts when these single cells are plated onto poly-L-ornithine/fibronectin-coated coverslips in culture medium supplemented with various growth factors (neurobasal medium supplemented with B27 supplement, 10 ng/ml NT3 (Neurotrophin 3), and 10 ng/ml BDNF (Brain-derived neurotrophic factor)). The aim is to have mature and immature neural cell types present in the final cell population, to be able to distinguish the different phases of neural development individually, and to always have a constant ratio between the different cell types occurring in the final culture.

An adaptation of the protocol above, based on a protocol used for the formation of dopaminergic neurons [60], has been used by De Groot and co-workers to differentiate ES-D3 stem cells into the cell types present in the CNS. In 23 days of culturing, the embryonic stem cells appear to differentiate into mature and immature neurons, astrocytes and oligodendocrocytes since cells express markers of mature neural cells (neurons (βIII-tubulin), astrocytes (GFAP) and oligodendrocytes (O4)) as well as markers of neural progenitor cells (NG2, A2B5, nestin). In addition, the stem cell assay indeed recapitulates in vitro the important neurodevelopmental processes of proliferation, migration and differentiation [28–32,62].

3.2. Human models

Based on the criteria described above, many hNPC models currently in existence could be utilized in screens for chemical effects on processes important for nervous system development. However, few of these models have been utilized for such purposes to date. Models of neural cells that can be regenerated in perpetuity without losing genotypic and phenotypic stability can be derived from a variety of different sources. Embryonic stem cells (ESCs) represent a likely source. There are currently 21 available cell lines approved by the NIH, several of which can be differentiated into neuroprogenitor/neuronal cells (NIH Human Embryonic Stem Cell Registry, 2008). Table 2 presents the link to the NIH stem cell registry as well as other links relevant to human stem cells.

In addition to these approved ESC lines, cells derived from fetal tissue [13,25,91,96,103,120] and adult neocortex [95] could also be used for screening approaches. Two of these hNPC lines are v-myc immortalized [13,25], retain karyotypic stability across many passages, and can be differentiated into both glial and neuronal subpopulations upon growth factor removal. hSN12W-TERT cells, a hNPC line derived from human fetal ventral telencephalon were immortalized using overexpression of human telomerase reverse transcriptase. They maintain high telomerase activity and a normal diploid karyotype after more than 40 passages [120]. This line expresses the hNPC markers nestin, vimentin, and SOX2, and differentiates into primarily GABAergic neurons, astrocytes, and oligodendrocytes. Upon growth factor removal, these cells were post-mitotic and displayed electrophysiological activity [120]. Rieske et al. [96] reported a population of GFAP-positive cells derived from fetal human brain parenchyma that expresses nestin and βIII-tubulin. These cells differentiate into both neurons and glia upon growth factor removal, but their ability to be subcultured and their stability after passaging remain to be determined. Other potential hNPC models that can be differentiated into multiple neuronal subpopulations include cells derived from human bone marrow stem cells [6,59,102], bone marrow stromal cells [67], adult skeletal muscle [101], and cord blood [12,97]. Thus, despite the limited use of hNPCs in screens for developmental neurotoxicity to date, there are many potential human-derived NPC models that could be evaluated for this purpose.

Table 2
Web resources for stem and neuroprogenitor cell cultures and research.

Organisation	Website
NIH Stem Cell Information	http://stemcells.nih.gov/research/current.asp
National Human Neural Stem Cell Resource	http://www.nhnscr.org/
International Stem Cell Forum	http://www.stemcellforum.org/
UK Stem Cell Bank	http://www.mrc.ac.uk/Ourresearch/Resourceservices/Stemcellbank/index.htm

One aspect that may facilitate utilization of stem and neuroprogenitor cells for screening purposes is commercial availability. Isolation and maintenance of stem or neuroprogenitor cells from rodents is labor intensive and expensive for many researchers. The advantage of commercially available cells is that they are easily obtained and often have been characterized by the distributing company in terms of the ideal culture conditions, the genomic stability, the phenotypes expressed after differentiation and the conditions necessary for differentiation. While this does not excuse the individual researcher from confirming the characteristics of the cells, it does save the labor and costs associated with the initial isolation, characterization and expansion of the cells. A brief discussion of a number of commercially available stem or NSC models follows.

Embryonic neural (EN)Stem-A cells (Millipore, Inc.) are a hNPC line derived from the NIH-approved H9 human embryonic stem cell line [111], which has a normal female (46XX) karyotype. ENStem-A cells express the NPC markers nestin and SOX2 (Breier and Shafer, unpublished). According to the supplier, ENStem-A cells also differentiate into multiple neuronal subtypes, including cholinergic, dopaminergic, and GABAergic neurons, glia, and oligodendrocytyes, and maintain a stable karyotype for at least 10 passages. ENStem-A cells have not been immortalized or otherwise transformed, and therefore the potential caveats of transformation are not at issue when using these cells. They also grow as a monolayer without fibroblast support. Thus, it may be a useful model for HCS/HTS approaches as direct effects of chemicals on these hNPCs can be assessed. To date, there have been no reports of utilization of this line in screening approaches, although they do attach to three dimensional scaffolds that could be used for HTS approaches and differentiate into neuronal phenotypes [19].

One hNPC model that has been used in HCS/HTS for chemical effects on developmental neurotoxicity is ReNcells isolated by ReNeuron Group (Guildford, Surrey, United Kingdom). Two ReNcell lines, CX and VM, are available commercially (Millipore) and are self-renewing, multipotent and phenotypically and genotypically stable due to immortalization with *myc* oncogene [34]. ReNcell CX cells were derived from a 14-week sample of human fetal cortex and proliferate linearly in monolayers. These cells have a normal male karyotype (46XY), express the neural progenitor markers nestin and SOX2, and differentiate into neuronal, astrocytic, and oligodendrocytic cell populations upon growth factor removal [34].

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ReNcell VM cells were derived from the ventral mesencephalon of 10-week fetal neural tissue, also have a normal male karyotype, express the neural progenitor markers nestin and SOX2 and proliferate linearly in a monolayer. ReNcell VM cells can be differentiated, not only into three neural subpopulations, but also into primarily dopaminergic cells that express tyrosine hydroxylase [34]. Thus, these cells may be useful as models to study dopaminergic neurodegeneration in addition to screening for potential developmental neurotoxicants. Neurons differentiated in ReNcell VM cultures also have been demonstrated to exhibit TTX-sensitive Na^+-currents and fire action potentials [34]. This demonstrates that these cells are electrically active and indicates the possibility of synapse formation and development of functional neural networks in differentiated ReNcell VM cell cultures, although this remains to be verified. ReNcell CX cells have been used in HTS assays for proliferation, and yielded promising results ([9]; see below). While ReNcell cells offer the advantages of human origin and unlimited availability, they still bear the limitations of myc-transformed cell lines. As with many of the models discussed in this review, further characterization of both the CX and VM lines is necessary to understand fully their utility in screening approaches for developmental neurotoxicity.

3.3. NPCs growing as neurospheres

In 1992, Reynolds, Weiss and colleagues [88,89] demonstrated for the first time that cells from the central nervous system (CNS) of adult and embryonic mice can be isolated and propagated in culture. In the presence of epidermal growth factor such NSC give rise to cell agglomerations that are termed 'neurospheres'. Furthermore, neurospheres can also be generated from single NSC [87] indicating that they have the potency for self renewal. The growth of similar human CNS precursors has recently been reported [11,17,75,106]. These hNPCs can be enzymatically dissociated and cultured after passaging, resulting in a monolayer of cells which are nestin-positive and contain subpopulations of cells that are GFAP- or βIII-tubulin-positive, and produce neurites in the presence of growth factors [94]. While they have been reported to be useful for only a single-passage, these hNPCs retain various neuronal and astrocytic markers beyond ten passages (Simpson, P; personal communication). Alternatively, human neurospheres can be further propagated by physical dissociation or 'chopping' to produce new neurospheres [108]. With this passaging technique, human neurospheres can be kept in culture for 3 to 4 months (Moors, M and Fritsche, E: unpublished observation).

Neurosphere cells are not synchronized, which is displayed by the presence of all phases of the cell cycle within spheres of human embryonic or fetal origin [5,72]. These cell cycle analyses or a clonal expansion assay show that only a small number of cells within a neurosphere are capable of proliferation (2.4 % and 2.25 % for mouse and human fetal spheres, respectively [72,87]).

Neurospheres are morphologically and functionally heterogeneous cell populations expressing stem cell markers, the neural progenitor marker nestin as well as markers of more mature neural cell populations like neurons and glial cells [7,54,66,69,72,82,83,86,90]. These different cell types are not evenly distributed throughout the sphere, but follow a regional distribution as shown for murine [16,71] and human [72] spheres. Nestin-positive undifferentiated cells are located in the periphery of the sphere, while differentiated neuronal (βIII-tubulin$^+$) and glial (GFAP$^+$) cells reside in the center. This dissemination is probably due to a growth factor gradient from the outside to the inside of the sphere. It is suggested that a similar gradient is responsible for NSC residing in the subventricular zone of the brain in vivo and that thus neurospheres mimic the in vivo situation in the brain in vitro. This notion is further supported by the following observations (i) that the proliferative capacity and differentiation potential of neurospheres after exposure to different external factors vary in a manner that reflects the developmental stage of the donor [52,112]; (ii) that the expression of many developmental control genes is maintained in a regionally specific manner in the neurosphere cultures after several passages, and the neurosphere-derived cells maintain the potential to differentiate into neuronal subtypes characteristic of their region of origin [47,48,61,78,80,82,118]; and (iii) that in neurospheres, as in the developing brain, several maturation stages of cell types co-exist [1,92]. While this cellular heterogeneity may be more reflective of in vivo nervous system development, this aspect of neurospheres must also be considered when developing and interpreting assays to detect effects of chemicals on developmental processes.

Upon growth factor withdrawal, neural progenitor cells migrate radially out of the sphere onto a given extracellular matrix (ECM) and thereby differentiate into cells expressing neural and glial markers which form the migration area [7,38,56,72,73,82,108,119,121]. At the same time, the zonal pattern inside the sphere disappears. After eight days of differentiation, βIII-tubulin$^+$ cells are found at the edge of the sphere, while nestin$^+$ and GFAP$^+$ cells are homogenously distributed throughout the sphere [72]. This means, that upon growth factor withdrawal, the sphere reorganizes itself. Furthermore in all likelihood, the migration area is not only formed by cells newly differentiated from nestin$^+$ precursors, but also by already differentiated cells which have migrated out of the sphere center. The migration area of human neurospheres seems to resemble the physiological distribution of brain cells in humans [4], as neurospheres contain a ratio of approximately 10% neuronal and 90% glial cells in the migration area after 2 days of differentiation [73]. With regard to neuronal subpopulations, RT-PCR analyses revealed that neurospheres express the GABA producing enzyme glutamic acid decarboxylase, the acetylcholine-synthesizing choline acetyltransferase, the dopamine-synthesizing DOPA decarboxylase and the serotonine producing tryptophane hydroxylase-1 as well as different members of NMDA receptors indicating that the major neuronal subtypes are present in differentiating human neurospheres (Moors, M and Fritsche, E: unpublished observation). Furthermore, immunofluorescence microscopy by Reubinoff et al. [86] revealed that differentiated cultures contain neuronal subtypes that synthesize glutamate, express glutamic acid decarboxylase, synthesize GABA and serotonin, and express tyrosine hydroxylase.

The process of migration is regulated by intracellular as well as extracellular stimuli. That mitogen activated protein kinase (MAPK) signaling determines normal neuronal migration is well described [49]. Fritsche and colleagues have recently demonstrated that migration of human neural progenitor cells out of the neurosphere is controlled by the MAPK ERK1/2-dependent and -independent pathways, while differentiation appears not regulated by these pathways [73]. Additionally, the extracellular matrix controls migration. Laminin matrices enhance migration, expansion, differentiation and elongation of murine [58] and human [37] neurosphere-derived neurons. Furthermore, human neural progenitor cell migration is prevented on collagen, fibronectin and poly-L lysine matrices (Fritsche E., unpublished observation) signifying a crucial role for the ECM in neural migration not only in vivo, but also in vitro.

Little is known about the similarities and specific differences of neurospheres generated from different species. However, Svendsen et al. have shown that rat and mouse neurospheres, grown under identical conditions, have very different long term expansion potentials with the rat cells entering senescence within 3–4 weeks of expansion [107]. No comparative human data are available, so far. On the contrary, laminin had similar effects on both human and mouse neural progenitor cell migration and differentiation, demonstrating the evolutionary conservation of regulation through laminin-binding integrins [37]. Although both mouse and human spheres show zonal distribution of cells within the sphere [16,72], there are some differences in differentiation between rodents and humans. While rat and human oligodendroglial progenitor's maturation in vitro follows the same pathway, human development takes twice as long as their rodent counterparts [119]. These few data suggest that there are basic similarities between rodent and human neurospheres. However, one has to consider possible species-specific differences

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depending on which aspect of neurodevelopment is examined. These differences need to be characterized in more detail in the future.

Taken together, neurospheres are three dimensional, heterogeneous, self-regulated cellular systems, which mimic basic processes of brain development in vitro: proliferation, migration and differentiation (Fig. 1). Commercial availability of human neurospheres (Normal Human Neural Progenitor Cells (NHNP cells), Cambrex, East Rutherford, NJ now Lonza, Verviers SPRL, Belgium) derived from 16 to 20-week old female or male whole brain homogenates has greatly facilitated the usage of neurosphere cultures for screening purposes.

4. Toxicity studies with rodent and human stem/progenitor cell models

4.1. Rodent

Murine NSCs have been used in several studies of developmental neurotoxicity, including the effects of endocrine disruptors [57], lead [50], methylmercury [77,109], and manganese [110]. Studies of Christoffer et al. [110] showed that NSCs exposed to toxic insults appear to be more sensitive than differentiated neurons, neuronal or glial cell lines, and point to NSCs as a valuable model for developmental neurotoxicity.

Moreover, De Groot and colleagues [28–32,62] are currently developing a rodent embryonic stem cell assay for systematic higher throughput developmental neurotoxicity testing that utilizes the mouse embryonic stem cell line ES-D3. Exposure of ES-D3 cells to methylazoxymethanol (MAM), methylmercury chloride (MeHgCl), and triiodothyronine (T3), developmental neurotoxicants which act on proliferation [35], migration [20,55], or differentiation [3], respectively, confirmed the sensitivity of these developmental phases to neurotoxicants. The effects of exposure to these developmental neurotoxicants observed in the in vitro assay were comparable with the effects observed in vivo (see e.g. [21] (MAM); [98] (references therein (MeHgCl)); [39] (T3)), i.e. MAM reduced proliferation, MeHgCl reduced migration and T3 stimulated oligodendrocyte differentiation in this in vitro assay.

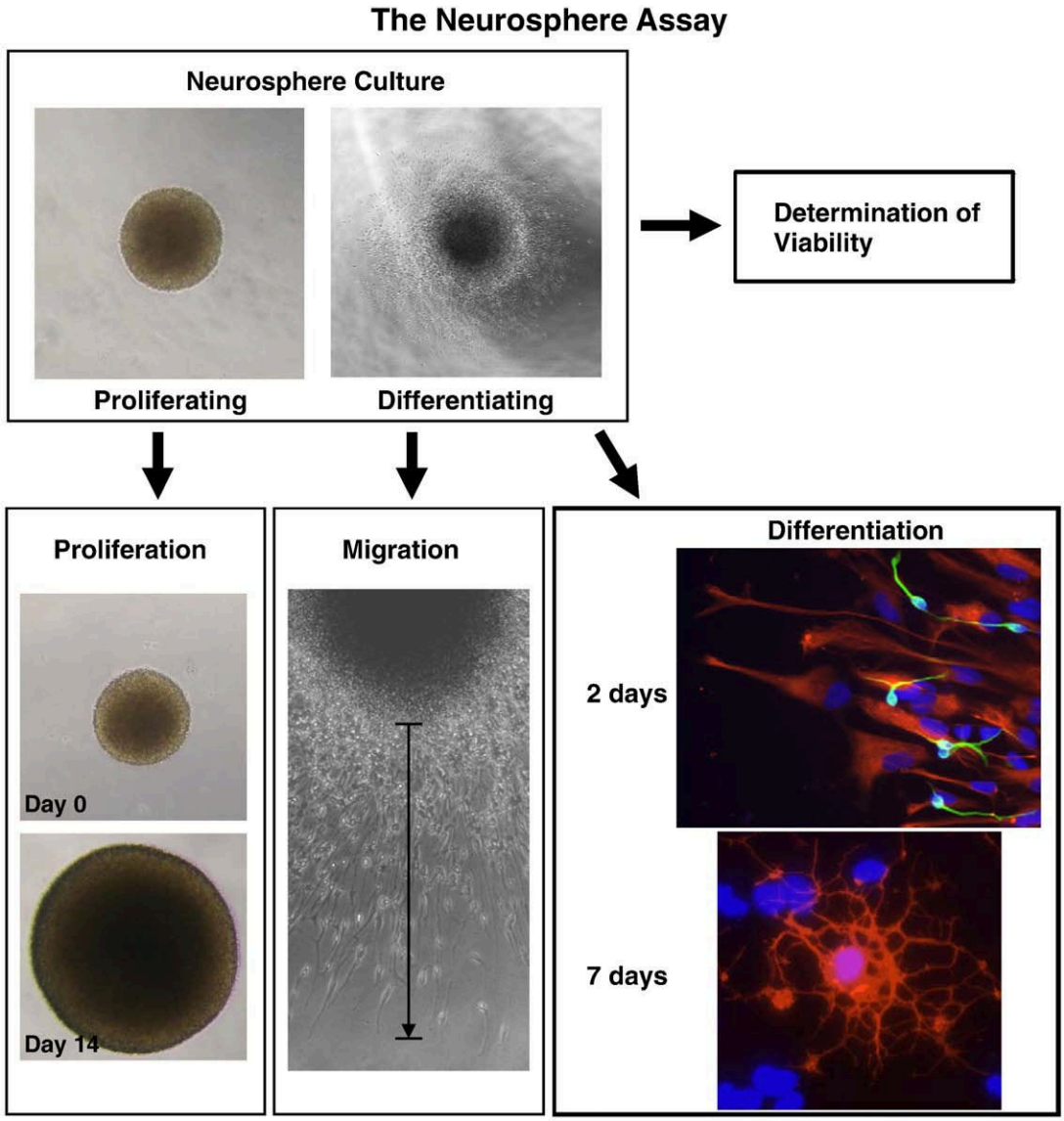

Fig. 1. Schematic overview over the 'Neurosphere Assay' for DNT testing utilizing human fetal neurospheres. Proliferation is assessed via increase in single sphere size or indirectly via increase in metabolic sphere activity. Upon withdrawal of growth factors and presence of an extracellular protein matrix, NPC migrate radially out of the sphere. Migration distance over time is measured as indicated by the arrow in 4 different positions of the sphere. Specific immunocytochemical staining of NPCs indicated the presence of βIII tubulin (green) and GFAP (red) after 2 days and O4 (red) after 7 days, respectively. In both the 2 and 7 day photos, nuclei are stained with DAPI and appear in blue. Viability of proliferating and differentiating neurospheres is assessed to differentiate between general cytotoxicity and specific developmental neurotoxicity of compounds. Pictures are provided by Kathrin Gassmann, Thomas Rockel and Timm Schreiber. See reference #78 for additional details.

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In addition to the characterization of the developmental phases and cell types recognized in this in vitro DNT assay, a method to quantify developmental neurotoxic effects is under development. The central hypothesis is that neurotoxicity can be determined by assessment of the numerical relationship between the resulting cell types in a compound treated culture as opposed to an untreated culture, but only when normalized to the total number of cells in the culture. For this, the (differentiated) cells are stained with a general Haematoxilin and Eosin (H&E) stain and are classified on the basis of their morphological features (Fig. 2 top)—i.e. a cell atlas typically for the DNT culture was developed—and quantified (Fig. 2 bottom) using stereological principles [44]. Conventional stains that are used worldwide, such as H&E staining, may be useful for the quantification of the cells in the culture; such staining procedures may yield highly reproducible results and be less expensive than advanced immunostains. The use of cell counting using stereological principles to determine developmentally neurotoxic effects has already been successfully used in in vivo developmental neurotoxicity [26,27]. Using the developed cell morphology atlas and counting method, results have shown that the cell class distribution based on morphological features was the same in 10 untreated cultures, collected over a period of 5 months (De Groot, unpublished observations). Since this proposed in vitro DNT assay has the phases of in vivo development of the CNS including differentiation into immature and mature neural cells, has a constant cell class composition and appears able to detect developmental neurotoxicity, this assay is a promising alternative for in vivo DNT studies in first tier screening settings. Further refinement and validation is necessary to guarantee continuity of the cell culture.

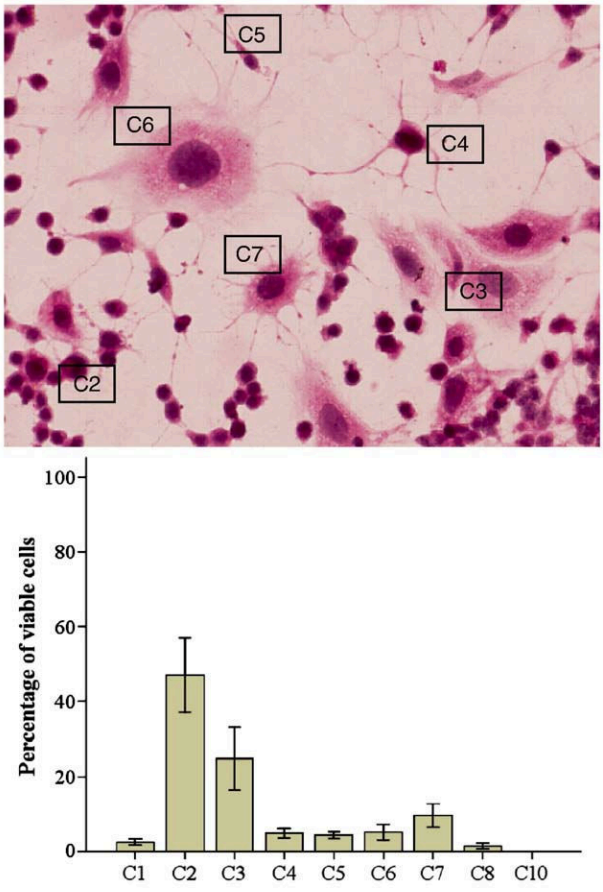

Fig. 2. Top: Fully differentiated cultures of ES-D3 embryonic stem cells stained with hematoxilin/eosin (H&E) reveal the presence of several different morphological cell types. A cell atlas was composed from such H&E stained cultures showing the different cell types (see indicated e.g. C2, C3, C4, C5, C6, C7). Bottom: To enable stereological microscopy cells were allocated to 10 different cell classes (C1–C10) based on the morphology of nucleus, cytoplasm, cell membrane, processes, cell shape and cell size: C1. Cells with 2 nuclei (not shown); C2. Small cells with dense nuclei and little cytoplasm; C3. Cells with vague boundaries present in groups; C4. Cells with multiple processes (>2) with dense cytoplasm (processes proceed from the cytoplasm); C5. Elongated cells; C6. Super sized cell (with 1 nucleus); C7. Medium sized cells with dense nuclei without long processes; C8. Cell mass (not shown); C9. Dead or apoptotic cells (not shown); C10. Cells with unknown morphology (not shown). Changes in the percentage of viable cells of each morphological type can then be assessed following exposure to neurotoxicants. Note that C9 does not appear in the graph with viable cells as C9 is the class with dead cells.

One advantage to using this ES-D3 stem cell model as part of an assay to detect developmental neurotoxicity is that ES-D3 cells are also used in the embryonic stem cell test (EST) for embryotoxicity [104]. In the EST assay, effects of toxicants on differentiation of ES-D3 cells into cardiomyocytes are compared to their effects on viability of ES-D3 and 3 T3 fibroblasts [41,42,105]. By letting the embryonic stem cells also differentiate into neural cells [36], the EST can be expanded to test for potential developmental neurotoxicants and this may reduce false negative results of the overall assay. With modified methods, such as using 96-wells plates, EST is more suitable for high-throughput [36,81]. However, one downside of this assay is that the long differentiation protocol (23 days) does not allow quick screening. Second, it leaves uncertainties in selecting appropriate chemicals exposure durations and concentrations. This is critical for in vitro testing because some lipophilic compounds (e.g. methylmercury, PCBs, PBDEs) may quickly accumulate in cells to concentrations 50–100 fold higher than their concentrations in the extracellular medium [68,74].

4.2. Human

A number of studies have utilized human neuroprogenitor cell models to study the effects of neurotoxicants. Ethanol is perhaps the most widely studied compound in neuroprogenitor cells, and has been reviewed elsewhere [76]. Only a few studies have focused specifically on developmental neurotoxicity and fewer still have focused on use of these models to develop assays for hazard identification and/or screening of potentially neurotoxic chemicals. Several of those studies utilized neurospheres, and will be discussed below in conjunction with studies utilizing neurospheres from rodents.

Studies from the laboratory of Noble and colleagues are notable because they have focused or included examination of chemical effects on glial progenitor function and/or glial differentiation. One study examined effects of chemotherapeutic agents (carmustine, cisplatin and cytosine arabinoside) on neuroprogenitor cells and oligodendrocytes and compared their cytotoxicity between these cell types and four glioblastoma lines (1789, UT-12, UT-4, T98). In general, the neuroprogenitor cells and oligodendrocytes were more sensitive to the chemotherapeutic agents than the glioblastoma cells [24]. In another study, methylmercury, lead and paraquat all induced pro-oxidative changes in oligodendrocyte precursor cells [65].

A recent report evaluated chemical effects on ReNcell CX proliferation and viability in a high-throughput/high-content format [9]. This work is being conducted as part of a larger project which is directed at development of HTS/HCS assays to detect chemical effects on processes important to development of the nervous system (e.g. proliferation, differentiation, neurite outgrowth, synaptogenesis) and is evaluating a number of different cellular models. The report demonstrated that known antiproliferative compounds inhibited ReNcell CX proliferation without effects on viability, and then tested effects on proliferation and viability of the cells in response to treatment with a set of sixteen chemicals for which evidence of developmental neurotoxicity in vivo does or does not exist. It was determined that, of the chemicals tested for which evidence of developmental neurotoxicity exists in vivo, 78% of them produced significant detriments on ReNcell CX cell proliferation and/or viability (although diphenhydramine reduced only viability by 7%, it was statistically significant). By contrast, only one (omeprazole) out of the

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seven chemicals for which there was no evidence of developmental neurotoxicity significantly inhibited cell proliferation and/or viability (Table 3). In the case of omeprazole, the effects on proliferation and viability were unexpected. However, Radio and co-workers reported that omeprazole caused significant changes in neurite outgrowth over the same concentration range [85]. Although there is no evidence for developmental neurotoxicity of this compound in vivo, it may nonetheless be a false positive compound in vitro. ReNcell CX cells have also been used recently to examine the effects of 320 pesticide (primarily) compounds on proliferation and viability [8]. While full analysis of the data is pending, this demonstrates the ability of this approach to screen large numbers of chemicals. Thus, ReNcell CX cells appear to be useful models for HTS assays, but such assays need additional evaluation using larger sets of chemicals containing known developmentally neurotoxic and inactive compounds.

4.3. Neurospheres

So far, research with neurospheres has largely focused on their application for neuroregeneration in disease states of the central nervous system [53,71,86,121]. Nevertheless, a few studies have also utilized neurospheres for toxicity studies in vitro by analysing a variety of endpoints such as viability, proliferation, migration, differentiation, neurite outgrowth and apoptosis. These provide support for their use in hazard identification screens for chemicals that may cause developmental neurotoxicity.

Richards et al. [93] screened two libraries of commercially available chemicals, one from Sigma-Aldrich (LOPAC library, 640 chemicals) and one from Tocris (259 chemicals), for their potential to stimulate neurite outgrowth from subpopulations of GFAP-positive (astrocytes) and Tuj1-positive (neuronal) hNPCs. The initial 1 μM screen yielded hit rates of 8.3 % (Tuj1+) and 3.0 % (GFAP+) for the LOPAC library, and 4.6 % (Tuj1+) and 9.3 % (GFAP+) for the Tocris library. A 'hit' was defined as a compound that increased neurite outgrowth index, form factor, and average neurite length per neuron by more than 35% of the control Platelet Derived Growth Factor response within the same plate [93]. Each hit was then further evaluated across a concentration range of 3 μM–30 nM.

All other studies did not systematically investigate the effects of a wide range of chemicals on developmental processes of neurospheres in vitro. Instead, they described effects of ethanol, lead, mercury or polychlorinated biphenyls—four of the five chemicals identified as developmentally neurotoxic for humans (see, [43])—on primary rodent or human neurospheres generated from different stages of gestation [14,18,46,50,70,84,100,114,115]. The outcomes of these studies are discussed in more detail in the following paragraphs.

Cell proliferation in a neurosphere can be determined in several different ways. BrdU or [methyl-^{3}H]-thymidine incorporation can be assessed with a scintillation counter or distribution of sphere cells within the cell cycle can be evaluated by FACS analyses [5,50,72,78,108]. Moreover, markers of proliferation, like Ki67, can be assessed in human and mouse spheres employing immunocytochemistry [2,12]. Proliferation of cells in neurospheres can also be assessed by counting the number of cells in dissociated spheres, measuring the increase in sphere diameter or measuring metabolic activity over time [56,72,86,108]. The association between these parameters is very high, indicating that each of these methods is suited to measure neurosphere proliferation [72]. The advantage of assessing single neurosphere proliferation by determination of increases in actual cell number within the sphere by the different methods indicated above lies in the consideration of compensatory mechanisms. These mechanisms are not taken into account by determining cell cycle phases or expression of proliferation markers. Several groups have investigated the influence of lead and ethanol on human and rodent neurosphere proliferation. Exposure of mouse E15 spheres to lead (0.01–100 μM) inhibited their proliferation in a concentration-dependent and brain region-specific manner [50]. Ethanol exerted differential effects on proliferation of human and rodent neurospheres. While 1–100 mM ethanol did not affect proliferation measured by cell cycle analyses or apoptosis in human neurospheres generated from CD133$^+$ cells isolated from fetal brains (GW 14 to 17; [114]), 70 mM ethanol decreased certain miRNAs causing increased BrdU incorporation in E12.5 mouse neurospheres [100], and 50–400 mM ethanol decreased proliferation as measured by sphere size in mouse E14.5 neurospheres generated from dissected ganglionic eminences [40]. In E15 rat neurospheres also prepared from the ganglionic eminences, 50 mM ethanol inhibited proliferation determined by [^{3}H]-thymidine uptake [115] or stimulated cell cycle activity (26 and 135 mM) in spheres prepared from rat E15 cortices [99]. These data may indicate species-specific and/or brain region-specific differences in the effects of ethanol on proliferation of human and rodent spheres, but additional studies are needed to confirm this difference.

Cell migration is easily assessed in neurospheres, because upon growth factor withdrawal NPCs start leaving the sphere in a 90° angle and their travel distance over time can be measured employing a phase contrast microscope. Moreover, migration speed of human fetal

Table 3
Effects of a set of 16 chemicals on proliferation and viability in ReNcell CX cells.[a]

Chemical	Evidence for developmental neurotoxicity[b]	Proliferation		Viability	
		Lowest effective concentration (μM)[c]	Percent inhibition	Lowest effective concentration (μM)	Percent inhibition
d-Amphetamine	+	0.01	20	–	–
Methylmercury	+	3	75	3	20
Cadmium	+	3	30	20	40
Lead	+	10	20	–	–
trans-Retinoic Acid	+	30	80	30	50
Dexamethasone	+	100	50	–	–
Diphenhydramine	+	–	–	100	7
5,5'-diphenylhydantoin	+	–	–	–	–
Valproic Acid	+	–	–	–	–
Omeprazole	–	30	30	100	40
Amoxicillin	–	–	–	–	–
Acetaminophen	–	–	–	–	–
Glyphosphate	–	–	–	–	–
Saccharin	–	–	–	–	–
d-Sorbitol	–	–	–	–	–
Dimethyl Phthalate	–	–	–	–	–

[a] From [9]. Chemicals were tested at concentrations ranging from 1 nM to 100 μM (except lead, which was not tested above 30 μM) in semi-logarithmic increments.
[b] For those chemicals with a plus symbol (+), a literature review yielded peer-reviewed publications reporting developmental neurotoxicity in mammals (see [9] for details).
[c] This column reports the lowest concentration at which a significant decrease (compared to vehicle controls) was observed for either proliferation or viability. The columns to the right report the percent inhibition of proliferation or viability, respectively at the lowest effective concentration.

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Table 4
Summary of *in vitro* models for developmental neurotoxicant screening.

Culture type	Relevance to DNT	Advantages	Limitations
Immortalized cell lines	Proliferating cells that can be differentiated into cells with neuronal characteristics	Human and non-human mammalian lines available Easy to proliferate and generate large numbers of cells Amenable to HTS/HCS Homogeneous cell population Most cell lines currently in use have been well characterized Inexpensive No animal use	Most are tumor-derived and may contain abnormal characteristics Not true neurons and in some cases not derived from neural tissue Increased chance of genomic instability with increasing passage number No three dimensional structure and lack of interactions between different cell types
Primary dissociated cultures	Can be prepared from various aged embryos and early (PND0-7) animals and from a variety of CNS regions.	Normal CNS tissue that recapitulates many aspects of development in vitro Extend true axons and dendrites Cultures can contain neurons and glia to allow interactions between different cell types to be studied Amenable to HCS/HTS Genomic stability not an issue	Neurons are post-mitotic, therefore proliferation and complete differentiation of cells cannot be studied. Requires a new set of animals for each culture. Very limited, if any, access to human cells
Pluripotent stem cells	Capable of forming any cell type within the organism and would allow the study of all aspects of nervous system formation	Both human and rodent models available Multiple cell types present in culture allows for interactions to be studied Reproducible and renewable source of cells	Maintaining stem cell colonies can be labor intensive and expensive Ethical considerations surround the use of human tissue derived from embryos Complete differentiation to neurons may require time and the conditions required to do so are not fully elucidated
Multipotent neuroprogenitor cells	Capable of forming any neuroectodermal cell type	Both human and rodent models available Multiple cell types present in culture allows for interactions to be studied Reproducible and renewable source of cells Monolayer cultures available that are amenable to HCS/HTS Easy to obtain and maintain cultures No animal use	Use of human NPCs from established, NIH stem cell lines considerably reduces ethical considerations Many aspects controlling differentiation have yet to be resolved. Many aspects of culture characteristics still unknown Unknown whether or not these models will provide better sensitivity to or predictivity of developmental neurotoxicity

NPC is very reproducible between assays and different individuals [72,73]. Two neurodevelopmental toxicants, ethanol and mercury, have been studied for their potential to interfere with normal NPC migration out of neurospheres. While 200 mM ethanol decreased the migration distance of NPC out of human neurospheres (GW 19) differentiated on poly D-lysin/laminin coated slides [73], 26 and 70 mM ethanol enhanced migration of mouse GD 12.5 NPC through the pores of laminin coated inserts of multi-well plates [15]. Whether these different results reflect true differences in migration speed in neurosphere assays between species after ethanol exposure or differences in experimental design needs further clarification. Furthermore, the effects of mercury compounds on human NPC migration have been investigated. Both MeHgCl (0.5–1 μM) and $HgCl_2$ (1–10 μM) inhibit migration without affecting viability [73].

Differentiation of post-migratory cells has been assessed after exposure to lead, ethanol, mercury compounds or PCBs. Lead caused a decrease in $MAP2^+$ neurons (0.1–10 μM) and $GalC^+$ oligodendrocytes (1–10 μM), while the number of $GFAP^+$ astrocytes increased (1–10 μM) in exposed mouse E15 spheres [50]. Exposure of rat E15 neurospheres to ethanol (10–50 mM) caused a decrease in $GFAP^+$ cells, while the number of $MAP2^+$ cells was not affected [14,70,115]. Effects of mercury compounds and polychlorinated biphenyls (PCB) on human neurosphere differentiation (GW 19) have also been investigated. MeHgCl (750 nM) and $HgCl_2$ (4 μM) reduced the number of βIII-tubulin$^+$ cells/total cells in the migration area [72]. By contrast, the non-coplanar PCB118 (1 μM) induced the number of generated oligodendrocytes/sphere, whereas the coplanar PCB126 exerted no effect [38].

In addition to the above studies performed in primary brain neurospheres other toxicity studies have been performed using neurospheres generated from the human immortalized embryocarcinoma cell line Ntera2/clone D1 [46]. Ntera2/D1 cells also have the ability to form neurospheres in culture which proliferate, migrate and differentiate under certain conditions into $TujIII^+$ and $GFAP^+$ cells. The authors used this model to test known human teratogens classed as non-embryotoxic (acrylamide), weakly embryotoxic (lithium, valproic acid) and strongly embryotoxic (hydroxyurea) for neuronal marker protein expression. Only valproic acid (500 μM) reduced the expression of the neuronal protein neuron-specific enolase (NSE), whereas valproic acid (125–500 μM) and acrylamide (25 μM) inhibited expression of TujIII [46], a neuron-specific protein. Additional characterization is needed to determine if this cell culture model, which has the clear advantage of the ability to generate essentially unlimited numbers of cells and hence neurospheres, is suited for developmental neurotoxicity testing.

Taken together, several studies indicate that the three dimensional neurosphere model is able to react on exposure to a variety of developmentally toxic agents. However, these studies have not tested systematically the predictivity of neurospheres for toxicity screens. Therefore, a defined number of positive and negative compounds must be tested in these cellular systems on the different endpoints: proliferation, migration, differentiation and neurite outgrowth. Moreover, a direct comparison of toxicant effects on neurospheres generated from the different species mentioned above will provide direct rodent to human comparisons that may decrease the uncertainty associated with animal to human extrapolations.

5. Research needs and future directions

The use of stem and neuroprogenitor cell models in neurotoxicology is an emerging field, with most publications occurring in the last 3–5 years. As such, there are a number of different areas in which additional research questions must be addressed and problems must

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be solved. This is particularly true if these models are to become useful in screens to detect chemicals with potential for developmental neurotoxicity and prioritize those chemicals for additional testing.

A significant amount of basic biology regarding stem and neuroprogenitor cells remains to be elucidated, and this impacts the ability to design reliable assays for neurotoxicity testing. While many of the models discussed have been demonstrated to differentiate into neurons and glial cells (astrocytes and oligodendrocytes), the cues responsible for differentiation are still not fully understood. This has several impacts on neurotoxicity screening. Until the cues for differentiation are better understood, the ability to control fully this process is diminished, and this may lead to greater inconsistencies in the process over time in the same lab or, more likely, differences across labs in how cells respond to toxicants. In addition, this also limits the ability of researchers to 'force' stem or NP cells along a specific pathway for differentiation and thus be able to examine effects of compounds on differentiation of neurons vs glia, or even more specifically on the differentiation of different neuronal subpopulations (e.g. cholinergic vs adrenergic vs gabaergic). It is anticipated that, as the field of neurobiology makes progress in understanding the cues for differentiation, this information will expand the potential for use of these cells in neurotoxicology for both mechanistic and screening studies.

With respect to screening compounds for the potential to cause developmental neurotoxicity, predictivity is a fundamental area that will be improved by additional research. To date, the number of compounds that have been examined in the screening approaches discussed here is relatively small, ranging from a few to less than 20 compounds. In order to adequately evaluate a screening method, additional studies must be conducted that examine chemical test sets containing greater numbers of compounds including both known developmental neurotoxicants and compounds for which evidence of developmental neurotoxicity in human and rodent models is lacking. This will allow determination of the rates of correct prediction of neurotoxicity as well as false positive and negative rates. One existing example of evaluation of a screening approach is the EST, which has been validated against a list of compounds that are known to be strong teratogens, weak teratogens and without evidence of teratogenicity [41,42]. Such an approach for in vitro developmental neurotoxicity screening will be facilitated by the availability of a literature review of the evidence for developmental neurotoxicity of over 300 compounds [79]. This will provide one source for developing a test set of compounds for evaluation purposes.

An underlying assumption in the use of stem and progenitor cells, particularly the use of those of human origin, is that these will be better models than currently available in vitro neuronal models. Indeed, these models do have a number of advantages or lack the limitations of other models (Table 4). However, the presumption that cells of human origin may be more sensitive than those from rodents has not been rigorously tested to date. Use of hESC or hNPC models may be more expensive than rodent cell models, and at the present time, there is a vast literature and knowledge base for many different rodent and human transformed cell lines. Thus, investigation of the relative sensitivities of ESC and NPC models compared to existing models should be part of the overall strategy to develop batteries of tests to screen compounds for the potential to cause developmental neurotoxicity and prioritize them for additional testing. This strategy is picked up by a currently running joint project funded by the German Ministry for Research and Education which evaluates the effects of a defined chemical test set on developmental performance (proliferation, migration, differentiation) of murine (Andrea Seiler, Berlin) and human (Martina Klemm, Andre Schrattenholz, Mainz) ESC, murine and human neurospheres (Ellen Fritsche, Duesseldorf) as well as the human teratocarcinoma cell line NT2 (Gerd Bickers, Hannover). Within this project, effects of chemicals on signals generated by neurosensorchips (Jan Giemsa, Rostock) are also compared between the different cell types. This project will identify differences in sensitivity towards the different chemicals depending on their age of generation and species. The aim is to develop a test battery for DNT testing that identifies threats to human brain development at different stages of development.

Besides predictivity of human toxicity, it is extremely important for an in vitro test system to be applicable for a certain throughput. While immortalized cell lines can be used in a high-throughput approach, the use of e.g. neurosphere models for toxicity testing has so far been considered a low-throughput approach. High-throughput systems as described by Xia et al. [117] for cytotoxicity testing would not be practical using neurospheres, because—especially for human spheres—it would be difficult to obtain the quantity needed on an ongoing basis. Moreover, spheres are larger in size than regular cell culture cells and thus need a certain cultivation space. As such, use of 1536-well plate formats is not possible. However, Collins et al. [23] recently proposed introducing alternative methods for toxicity testing. Besides the high-throughput testing methods with in vitro assays investigating effects of >10,000 chemicals/day using dissociated cell models, the authors describe medium-throughput testing in alternative animal models like zebrafish or drosophila, analysing 100–10,000 chemicals/year for their toxic potential. More complex three dimensional in vitro systems such as neurospheres could also be used to test for disturbances of organ-specific developmental processes such as migration and differentiation and should be included in this medium-throughput category. In a tiered testing approach the neurosphere assay could provide a more complex human system for developmental neurotoxicity testing. Culture, proliferation, migration and differentiation of human neurospheres in 96-well plates has been established in the laboratory of Fritsche and colleagues. Plating of individual spheres with a suitable robot as well as automated fluorescence and imaging analyses could make the neurosphere assays practical for medium-throughput analyses.

Conflict of interest statement

Nothing declared.

Note added in proof

Policies for federal funding of stem cell research in the United States changed when Executive Order (EO) 13505 was issued by President Barak Obama. This could dramatically change how NIH funds stem cell research. Details can be found on the NIH Stem Cell Information website listed in table 2.

Acknowledgements

Authors from the TNO Quality of Life thank Mariska Mulderij and Joost Bruijntjes for their skillful technical support. The work at the TNO (development of a quantitative in vitro assay for developmental neurotoxicity using ES D3 stem cells) was supported by a research grant from the Dutch Organisation ZonMw (File no. 114000077), by the Dutch Ministry of Health, Welfare and Sports and the Ministry of Social Affairs and Employment. The work of the IUF (murine/human neurospheres) was supported by research grants from the German Ministry for Research and Education, the Federal Institute for Risk Assessment (ZEBET), and the German Research Foundation (Graduate College 1427, project 3). The authors would also like to thank Drs. William R. Mundy and Sid Hunter, of the U.S. Environmental Protection Agency for their useful comments on a draft version of this manuscript.

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Please cite this article as: J.M. Breier, et al., Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: State of the science, Neurotoxicol. Teratol. (2009), doi:10.1016/j.ntt.2009.06.005

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Please cite this article as: J.M. Breier, et al., Neural progenitor cells as models for high-throughput screens of developmental neurotoxicity: State of the science, Neurotoxicol. Teratol. (2009), doi:10.1016/j.ntt.2009.06.005

2.2 Human Neurospheres as Three-Dimensional Cellular Systems for Developmental Neurotoxicity Testing

Moors M, Rockel TD, Abel J, Cline JE, **Gassmann K**, Schreiber T, Schuwald J, Weinmann N, Fritsche E.
Environmental Health Perspectives 2009 July;117(7) :1131-1138

Das sich entwickelnde Gehirn weist eine besonders hohe Sensitivität gegenüber schädigenden Einflüssen auf, die zu einer Beeinflussung der Gehirnfunktion führen können. Mehr als 10% der Kinder leiden an neurologischen Erkrankungen, die neben genetischen Faktoren auch auf eine Exposition gegenüber exogenen Noxen während der Gehirnentwicklung zurückzuführen sind, so dass eine Bewertung des entwicklungsneurotoxischen Potentials von Chemikalien zunehmend an Bedeutung gewinnt.
Außer kosten- und zeitaufwendigen Tierversuchen existieren bis heute jedoch keine validierten Testmethoden, die eine verlässliche Risikobewertung in Hinblick auf Entwicklungsneurotoxizität (ENT) erlauben. Ein alternatives *in vitro* Testmodell sollte die humane Gehirnentwicklung möglichst wirklichkeitsgetreu abbilden und Störungen der grundlegenden Prozesse der Gehirnentwicklung nach Einwirkung toxischer Noxen detektieren können.
Bei dem in dieser Publikation beschriebenen alternativen *in vitro* Testmodell handelt es sich um humane neurale Progenitorzellen, die als Neurosphären in Suspension kultiviert werden. Durch Entzug der Wachstumsfaktoren und nach Bereitstellung einer geeigneten Proteinmatrix werden die Migration der Zellen aus der Sphäre sowie die Differenzierung eingeleitet. Immuncytochemische Anfärbungen mit spezifischen Markern ergaben, dass es sich bei den migrierenden Zellen um eine sehr heterogene Zellpopulation handelt, die sowohl Marker für unreife als auch reifere neurale Subtypen enthält. Es können alle drei neuralen Subtypen Neurone, Astrozyten und Oligodendrozyten identifiziert werden und in Abhängigkeit von der Differenzierungszeit ist eine Reifung des Zellsystems feststellbar. Cryotomschnitte durch intakte Neurosphären unter proliferativen Bedingungen ergaben, dass Neurosphären auch im Inneren eine heterogene Zelltypverteilung aufweisen, die ihnen eine in vivo ähnliche Organstruktur verleit. Durch die Etablierung spezifischer Assays für Viabilität, Proliferation, Migration, Differenzierung und Apoptose mit endpunktbasierten Kontrollen und der Störung dieser Endpunkte durch bekannte entwicklungsneurotoxische Agenzien wie Methylquecksilberchlorid und Methylquecksilber können wir zeigen, dass humane Progenitorzellen kultiviert als Neurosphären ein erfolgsversprechendes Testmodell für ENT darstellen.

Die Verfasserin der Dissertation etablierte selbstständig sowohl den Proliferationsassay als auch den TUNEL-Assay für das Neurospärenmodell und führte die den entsprechenden Abbildungen zugrunde liegende Experimente durch. Darüber hinaus war sie an der Durchführung und Analyse der Cryotomschnitte beteiligt.

Human Neurospheres as Three-Dimensional Cellular Systems for Developmental Neurotoxicity Testing

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Background: Developmental neurotoxicity (DNT) of environmental chemicals is a serious threat to human health. Current DNT testing guidelines propose investigations in rodents, which require large numbers of animals. With regard to the "3 Rs" (reduction, replacement, and refinement) of animal testing and the European regulation of chemicals [Registration, Evaluation, and Authorisation of Chemicals (REACH)], alternative testing strategies are needed in order to refine and reduce animal experiments and allow faster and less expensive screening.

Objectives: The goal of this study was to establish a three-dimensional test system for DNT screening based on human fetal brain cells.

Methods: We established assays suitable for detecting disturbances in basic processes of brain development by employing human neural progenitor cells (hNPCs), which grow as neurospheres. Furthermore, we assessed effects of mercury and oxidative stress on these cells.

Results: We found that human neurospheres imitate proliferation, differentiation, and migration *in vitro*. Exposure to the proapoptotic agent staurosporine further suggests that human neurospheres possess functioning apoptosis machinery. The developmental neurotoxicants methylmercury chloride and mercury chloride decreased migration distance and number of neuronal-like cells in differentiated hNPCs. Furthermore, hNPCs undergo caspase-independent apoptosis when exposed toward high amounts of oxidative stress.

Conclusions: Human neurospheres are likely to imitate basic processes of brain development, and these processes can be modulated by developmental neurotoxicants. Thus, this three-dimensional cell system is a promising approach for DNT testing.

Key words: apoptosis, differentiation, DNT, human neurospheres, mercury, migration, proliferation. *Environ Health Perspect* 117:1131–1138 (2009). doi:10.1289/ehp.0800207 available via *http://dx.doi.org/* [Online 26 February 2009]

Developmental neurotoxicity (DNT) of environmental chemicals has been recognized worldwide as a serious threat to human health, and the resulting neurologic deficits negatively affect families and society (Goldman and Koduru 2000; Needleman et al. 2002). Current DNT testing guidelines (Organization for Economic Cooperation and Development 2007; U.S. Environmental Protection Agency 1998) propose investigations in rodents, mainly rats. Such a DNT *in vivo* testing strategy implies the use of 140 dams and 1,000 pups and is therefore extremely time- and cost-intensive (Lein et al. 2005). Relying solely on the existing guidelines to address current and anticipated future regulatory demands for DNT of the thousands of chemicals for which there are few to no DNT data would incur unacceptable costs in terms of animals and person-years (Lein et al. 2007). Therefore, according to the "3R principle" (reduction, replacement, and refinement) of Russel and Burch (1959), alternative testing strategies are needed to address animal welfare by refining and reducing animal experiments, and to create affordable, sensitive, and mechanism-based methods suitable for high- or medium-throughput screening (Collins et al. 2008). Furthermore, the inclusion of human-cell–based *in vitro* systems into an integrated DNT tiered testing approach has been recommended to circumvent species differences (Coecke et al. 2007).

To combine transatlantic strengths and avoid doubling of work, a partnership between the Johns Hopkins Center for Alternatives to Animal Testing (Developmental Neurotoxicity TestSmart program), the European Centre for the Validation of Alternative Methods, and the European Chemical Industry Council has been formed. This partnership follows the common goal of "incorporating *in vitro* alternative methods for DNT testing into international hazard and risk assessment strategies" (Coecke et al. 2007). Coecke et al. (2007) provided a comprehensive overview of the existing *in vitro* models and stated that, "although all the test systems described were not developed for regulatory purposes at this stage if they prove useful, we hope that this report will encourage their further development to render them amenable to high-throughput approaches."

Therefore, the aim of this work was *a*) to introduce the cell biological characteristics of human neurospheres as a three-dimensional cell system approach for DNT testing; *b*) to demonstrate that neurospheres are likely to mirror basic processes of brain development, such as proliferation, differentiation, migration, and apoptosis; and *c*) to demonstrate that these processes can be modulated by developmental neurotoxicants.

Materials and Methods

Chemicals. We obtained methylmercury chloride (MeHgCl) from Riedel-de Haën (Seelze, Germany); all other substances were obtained from Sigma Aldrich (Munich, Germany), unless otherwise stated.

Cell culture. Cryopreserved normal human neural progenitor cells (hNPCs; Lonza Verviers SPRL, Verviers, Belgium) were cultured at 37°C and 5% CO_2 as a suspension culture in proliferation medium consisting of Dulbecco's modified Eagle medium (DMEM) and Hams F12 (3:1) supplemented with B27 (Invitrogen GmbH, Karlsruhe, Germany), 20 ng/mL epidermal growth factor (EGF; Biosource, Karlsruhe, Germany), and 20 ng/mL recombinant human fibroblast growth factor (FGF; R&D Systems, Wiesbaden-Nordenstadt, Germany) (Moors et al. 2007). When spheres reached 0.7 mm in diameter, they were chopped up to passage 3 with a McIlwain tissue chopper. Differentiation was initiated by growth factor withdrawal in differentiation medium [DMEM and Hams F12 (3:1) supplemented with N2 (insulin, transferrin, sodium selenite, putrescine, and progesterone; Invitrogen)] and plated onto poly-D-lysine/laminin–coated chamber slides (BD Bioscience, Erembodegem, Belgium).

Chemical exposure. We exposed cells to indirubin (10 μM) in proliferation medium (28 hr), and to cAMP (200 μM), MeHgCl (250 nM to 1 μM), mercury chloride ($HgCl_2$; 500 nM to 10 μM, 48 hr) or staurosporine (0.1 and 1 μM), or hydrogen peroxide (H_2O_2; 0.1 and 1 mM) (24 hr) in differentiation medium. We chose concentration ranges of mercury according to Monnet-Tschudi

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Supplemental Material is available online at http://www.ehponline.org/members/2009/0800207/suppl.pdf

This work was supported by the German Federal Institute for Risk Assessment; the German Federal Institute for the Environment, Nature Conservation and Nuclear Safety; the German Federal Ministry for Education and Research (13925C); and the Deutsche Forschungsgemeinschaft (GK1427).

The authors declare they have no competing financial interests.

Received 19 September 2008; accepted 26 February 2009.

et al. (1996), who found a concentration of 1 μM to be cytotoxic.

Migration analyses. Migration analyses were performed as previously described (Moors et al. 2007). For living cell migration analyses, neurospheres were grown in the Focht Chamber System 2 (Bioptechs, Butler, PA, USA) under temperature- and CO_2-controlled conditions. Images were acquired every 2 min by a Zeiss Axiovert 100 inverted microscope (Zeiss, Goettingen, Germany).

Immunohistochemistry. Proliferating or differentiating spheres were fixed in 4% paraformaldehyde for 30 min. After washing spheres in phosphate-buffered saline (PBS), they were incubated in a 25% sucrose solution (wt/vol) overnight at 4°C. Afterward, spheres were transferred to tissue-freezing medium (Jung HistoService, Nussloch, Germany). Cryostat sections (10 μm) were prepared for immunohistochemistry. Antibodies for staining were nestin (1:150; BD Bioscience), glial fibrillary acidic protein (GFAP; 1:100, Sigma Aldrich), or β(III)tubulin (1:100; Sigma Aldrich).

Immunocytochemistry/differentiation analyses. We performed immunocytochemistry as previously described (Fritsche et al. 2005; Moors et al. 2007). For quantification analyses, we used the Metamorph analysis software package (version 7.1.7.0; Universal Imaging Corp., West Chester, PA, USA). We determined the variation of protein expression by relating area of fluorescence signal to cell number in a region of interest within the migration area. Individual pixels were identified as "positive" if the fluorescence signal exceeded a determined color threshold [green, hue (H) 71–113, saturation (S) 10–255, intensity (I) 10–255; yellow/red, H 0–71, S 10–255, I 10–255)]. To determine the cell number, we selected images for positive 4′,6-diamidino-2-phenylindole (DAPI) staining (blue, H 152–180, S 10–255, I 10–255) and morphologic parameters (integrated morphology analysis: area, $10^4 > n > 10^7$; ellipsoid form factor, $0.1 > n > 1.8$).

Fluorescence-activated cell sorting (FACS)/cell cycle analyses. Proliferating neurospheres were grown in proliferation media with or without growth factors or exposed to indirubin. To obtain a single-cell suspension, neurospheres were washed once in PBS, incubated with Accutase (100%; PAA, Cölbe, Germany) at 37°C for 20 min, and then gently pipetted. The suspension was centrifuged (4°C, 1,400 × *g*, 5 min) and the pellet was resuspended in PBS containing 0.8% paraformaldehyde (Polyscience Inc., Eppelheim, Germany). Cells were fixed for 30 min at 4°C, centrifuged (4°C, 1,400 × *g*, 5 min), resuspended in PBS containing 0.15% saponin and 10 μg/mL RNase, and incubated for 30 min at 37°C. We then added 50 μg/mL propidium iodine 5 min before FACS analyses.

Cell viability, apoptosis, and proliferation assays. We measured cell viability using the CellTiter-Blue assay (Promega, Mannheim, Germany) as previously described (Moors et al. 2007). The assay is based on measurements of the mitochondrial reductase activity by conversion of the substrate resazurin to the fluorescent product resorufin by mitochondrial reductases, which can be assessed in a fluorometer. The lactate dehydrogenase (LDH) assay (CytoTox-One; Promega) assesses cell death by measuring LDH that leaks out of dead cells into the media. We performed the assay according to the manufacturer's instructions. Briefly, supernatants of treated cells were collected and incubated with an equal amount of CytoTox-One reagent for 4 hr before the detection of fluorescence (excitation, 540 nm; emission, 590 nm). Caspase-3/-7 activities were measured with the Apo-One Kit (Promega) according to the manufacturer's instructions. Briefly, cells were lysed and caspase activity was assessed by measuring the cleavage of a caspase-3/-7–specific fluorescent substrate (Z-DEVD-R110) with a fluorometer (excitation, 488 nm; emission, 538 nm).

For proliferation assays, spheres were cultured in proliferation medium supplemented with or without 20 or 100 ng/mL EGF in 96-well plates. We assessed cell viability as a measure for cell number using the CellTiter-Blue assay at different time points. Because the dye caused no acute cytotoxicity, spheres were washed with medium after fluorescence reading and then the same spheres were monitored over a period of 2 weeks. For determination of sphere size, we gauged sphere diameter optically with an object micrometer. We counted the number of cells/sphere after trypsination (0.25% trypsin; Invitrogen) for 2 min.

TUNEL assay. For terminal deoxynucleotidyl transferase 2′-deoxyuridine 5′-triphosphate (dUTP) nick end labeling (TUNEL) assays, we used fluorescein-coupled dUTP and the terminal transferase kit from Roche Diagnostics (Mannheim, Germany) to label DNA strand breaks; nuclei were counterstained with Hoechst 33258 (Invitrogen). Plated neurospheres were exposed to staurosporine (1 μM) or H_2O_2 (1 mM) after 48 hr of differentiation. After another 12 and 24 hr, cells were fixed with 4% paraformaldehyde, washed twice with PBS, covered with reaction mixture (2.5 mM $CoCl_2$, 5 μM fluorescein coupled dUTP, 5,000 U/mL terminal-transferase, 2 μg/mL Hoechst, 0.1% triton in 1× terminal transferase buffer), and incubated at 37°C for 1 hr. Slides were then washed with PBS three times and mounted with PBS/glycerol (1:1). Stained cells were analyzed with a fluorescence microscope.

Statistics. We used analysis of variance combined with the Bonferroni post hoc test for multifactor analyses (time and concentration effects), and the Student's *t*-test for two-group comparisons (treatment vs. control; two time points). The significance value was set at $p < 0.05$. To describe the associations between independent variables (diameter/cell number; diameter/fluorescence), we fitted curves up to the third degree. We used R^2 as a measure of goodness of fit.

Results

Human neurospheres grow floating freely in defined medium without addition of serum [see Supplemental Material, Figure 1A (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)]. Upon withdrawal of growth factor, cells migrate radially out of the sphere onto a poly-D-lysine/laminin matrix, thereby forming a migration area [see Supplemental Material, Figure 1B and the video (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)]. Each cell leaves the sphere edge in a 90° angle and travels away in a straight line. Moreover, cells move toward and away from each other.

To evaluate reproducibility and stability of neurosphere migration, we assessed dependence of migration speed on neurosphere size. Therefore, the distance between the sphere edge and the farthest outgrown cells was measured 24 hr after plating, dependent on different sphere diameters. Supplemental Material, Figure 1C (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf) shows that spheres with a diameter between 0.2 and 0.7 mm wander approximately 0.48 mm within 24 hr (e.g., 0.2 mm diameter, 0.48 ± 0.06 mm; 0.7 mm diameter, 0.48 ± 0.09 mm; mean ± SD), demonstrating that migration speed is independent of sphere size. Moreover, cells from different donors (0.3-mm-diameter spheres) also did not vary significantly in migration speed over 24 hr [see Supplemental Material, Figure 1D (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)].

Next, we analyzed the cellular composition of neurospheres. We sliced 10-μm cryostat sections of proliferating neurospheres and examined expressions of *a*) nestin, a marker protein for neural stem cells; *b*) β(III)tubulin, which stains neurons; or *c*) GFAP for glial cells. Immunocytochemical analyses revealed nestin-positive (+) cells were located mainly in the sphere periphery, whereas β(III)tubulin$^+$ and GFAP$^+$ cells resided in the sphere center (Figure 1A,B). This pattern disappeared after spheres were plated for differentiation. After 8 days of differentiation, β(III)tubulin$^+$ cells were located at the edge of the sphere, whereas nestin$^+$ and GFAP$^+$ cells were homogenously distributed throughout the sphere (Figure 1C,D).

In addition to the sphere itself, we investigated the cellular composition of the

migration area after 24 hr and 7 days of migration. Twenty-four hours after plating, nearly all migrated cells seemed to express nestin, showing that immature cells migrate out of the sphere. Furthermore, β(III)tubulin$^+$ and GFAP$^+$ cells were also located in the migration area. In contrast, 7 days after plating almost all cells lost nestin expression and became β(III) tubulin$^+$ or GFAP$^+$ (Figure 2A). Quantification of the number of pixels in the respective images revealed a 5.5-fold reduction in the number of nestin$^+$ pixels/nuclei after 6 more days of differentiation (570.9 ± 64 to 103.2 ± 29 pixels/nuclei; mean ± SD), whereas in the same time period the number of β(III)tubulin$^+$ and GFAP$^+$ pixels increased 4.7- and 1.9-fold, respectively [β(III)tubulin, from 118.7 ± 27.4 to 509.6 ± 55 pixels/nuclei; GFAP, from 250.8 ± 56 to 480.7 ± 198 pixels/nuclei; Figure 2A]. Furthermore, the immunocytochemical staining for β(III)tubulin suggests that neuronal cells may form connections and thus build neuronal networks (Figure 2A).

Another cell type emerging from neural precursor cells are O4$^+$ oligodendrocytes. They form within the neurosphere (Fritsche et al. 2005) and migrate out of the sphere over time. After 2, 4, and 7 days of differentiation, 3 ± 0.2, 52 ± 1, and 210 ± 5 O4$^+$ cells (mean ± SD), respectively, were located in the migration area (Figure 2B). They also changed morphology over time. Although after 48 hr most O4$^+$ cells were bipolar, we found more branching after 4 days; after 7 days of differentiation, multipolar and membrane sheet-forming cells were prominent (Figure 2C).

Next, we developed assays that identify changes in cell proliferation, differentiation, migration, and apoptosis by applying model chemicals, which are known to interfere with normal brain development (Grandjean and Landrigan 2006).

Cell proliferation in a neurosphere can be determined by counting the number of cells per dissociated sphere or by measuring the increase in sphere diameter over time. Figure 3A shows that there was a very good association between these two parameters

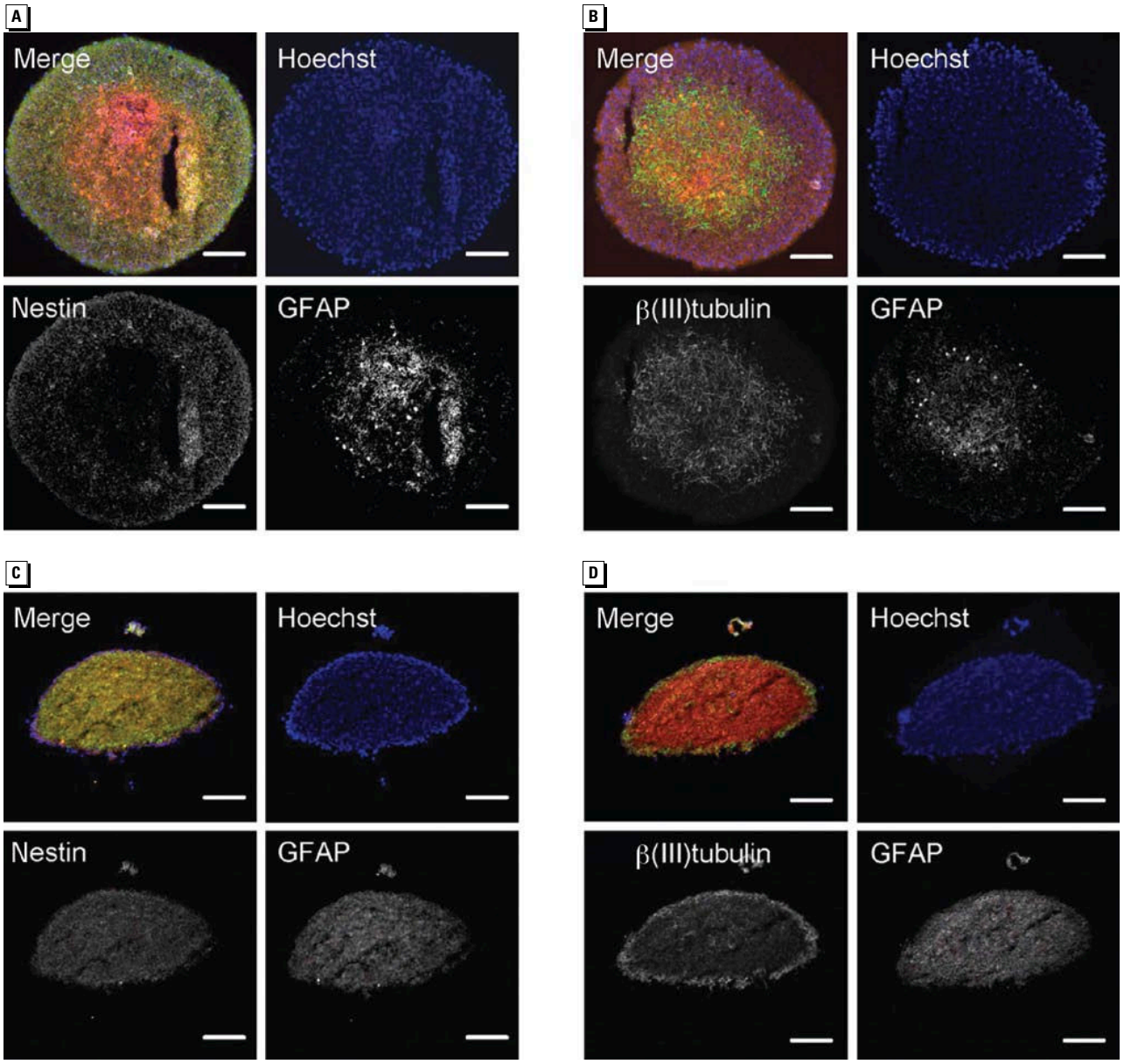

Figure 1. Cellular composition of human neurospheres shown in cryostat sections (10 µm) of proliferating (*A* and *B*) and differentiating (8 days after plating; *C* and *D*) neurospheres (representatives of five spheres for each developmental stage). Nuclei are stained in blue with Hoechst; nestin and β(III)tubulin are stained in green; and GFAP is stained in red. Individual antibody stainings are shown as contrast images. Bars = 100 µm.

(e.g., 2.6 × 10^3 and 5.3 × 10^4 cells for spheres 0.3 and 0.6 mm in diameter, respectively). We verified this observation and made the method suitable for high-throughput analyses by measuring viability of neurospheres dependent on sphere diameter with the CellTiter-Blue assay. Figure 3B demonstrates that viability of spheres correlates well with their sizes (e.g., spheres 0.3 and 0.6 mm in diameter had 4 × 10^3 and 8 × 10^3 relative fluorescence units, respectively). Growth of neurospheres in the absence or presence of 20 or 100 ng/mL EGF caused a 1.5 ± 0.4-fold or 2.4 ± 0.3-fold induction in viability (mean ± SD), respectively over 14 days, although the same spheres gained 0.08 ± 0.03 or 0.2 ± 0.04 mm in diameter, respectively, during this time (Figure 3C,D). We observed no differences in proliferation between spheres grown in EGF in the presence or absence of FGF (data not shown). Cultivation without growth factors as a negative control did not change size or viability. Thus, this assay can assess sphere proliferation.

We verified these data by FACS analyses for DNA content using propidium iodine staining. Among all stained cells, 97.75% showed only a G_0/G_1 peak (Figure 3F), whereas we found a typical cell cycle distribution for proliferating cells in only 2.72% of the population. About 35% of these were in G_2/M or S phase (Figure 3F, control), suggesting fast cell-cycling behavior. Indirubin, a G_2/M blocking agent that blocks signaling of cyclin-dependent kinases (Hoessel et al. 1999), increased the cell fraction in G_2/M phase from 14% to 37.8% (Figure 3F), whereas withdrawal of growth factors caused G_1 arrest.

To investigate effects of chemicals on differentiation, we exposed neurospheres to different Hg compounds. Immunocytochemical analyses after 48 hr revealed that migration areas of control cells consist of 10% β(III)tubulin$^+$ cells and 90% GFAP$^+$ cells (Moors et al. 2007). MeHgCl (500 and 750 nM) reduced the amount of β(III)tubulin$^+$ cells to 8 ± 0.17% (mean ± SD) and 2.3 ± 0.57%, respectively. Exposure to 4 µM $HgCl_2$ decreased the number of β(III)tubulin$^+$ cells to 4.7 ± 2.3%. In contrast, cAMP increased the formation of β(III)tubulin$^+$ cells to 165.4 ± 9% of control cells (Figure 4).

Next, we investigated the effects of Hg on cell migration with the neurosphere migration assay (Moors et al. 2007). Exposure to MeHgCl (500 nM) caused an inhibition of cell migration to 78.7% ± 7% of control values, which was further reduced by higher MeHgCl concentrations. $HgCl_2$ (4 µM) also reduced cell migration to 73.6 ± 13% of the controls (Figure 5A, B). Notably, cell migration was significantly affected by noncytotoxic Hg concentrations (Figure 5C).

To determine whether human neurospheres can be stimulated to undergo apoptosis, we exposed them to staurosporine, a potent inducer of the intrinsic apoptotic pathway via cytochrome c release followed by activation of the caspase cascade (Slee et al. 2000), or H_2O_2, a direct reactive oxygen species (ROS) donor, for 24 hr. LDH measurements of neurosphere supernatants indicate that staurosporine and H_2O_2 induce cell death in a concentration-dependent manner. However, the human neuroblastoma cell line SH-SY5Y (ATCC, Wesel, Germany) is more susceptible to staurosporine- and H_2O_2-induced LDH release than are the spheres, as indicated by a higher LDH release at lower concentrations, which we confirmed using phase-contrast microscopic images (Figure 6A,B). To explore whether staurosporine (1 µM) or H_2O_2 (1 mM) induced cell death via apoptosis, we performed TUNEL assays. Although the basal apoptosis rate of hNPCs after 3 days of differentiation was approximately 1% (data not shown), both treatments induced TUNEL-positive cells, showing that apoptosis is involved in staurosporine-induced and H_2O_2-induced cell death (Figure 6D). However, measurements

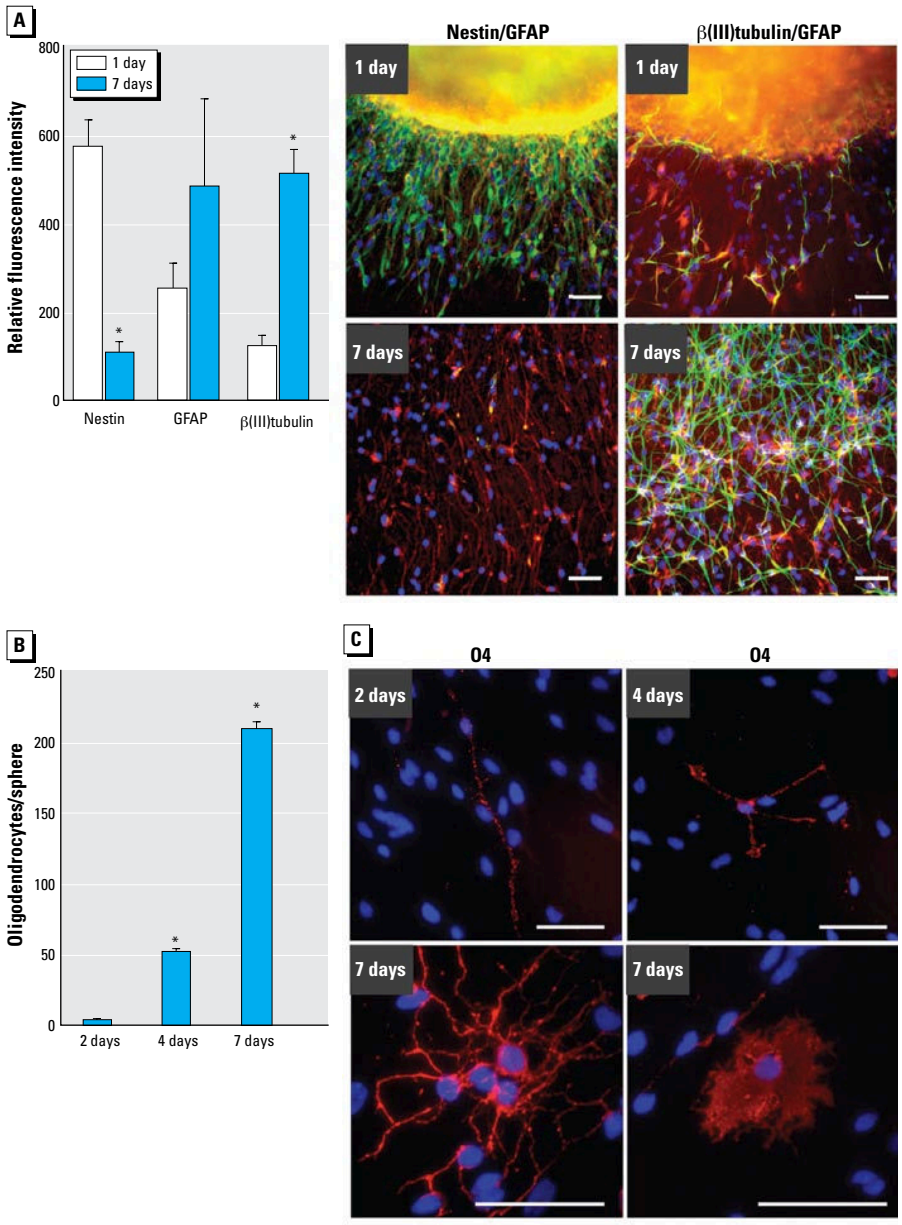

Figure 2. Cellular composition of the neurosphere migration area. (*A*) Quantification of nestin$^+$, GFAP$^+$, and β(III)tubulin$^+$ pixels after 1 and 7 days of differentiation (mean ± SD) and representative photographs [left: nestin (green), GFAP (red); right: β(III)tubulin (green), GFAP (red)]. Ten individual spheres were included in each calculation. (*B*) Quantification of migrated O4$^+$ cells, counted manually after 2, 4, and 7 days of differentiation in six individual spheres (mean ± SD). (*C*) Morphology of O4$^+$ cells at different time points. Bars = 100 µm.
*$p \le 0.05$.

of effector caspase-3/-7 activities indicate that staurosporine-induced cell death is caspase dependent, whereas H_2O_2-triggered cell death is caspase independent (Figure 6C).

Discussion

In humans, DNT results in learning deficits and mental retardation (Hass 2006; Schettler 2001). Furthermore, various clinical disorders (e.g., schizophrenia, autism) are results of interference with normal brain development, and their etiologies are suspected to also imply environmental components (Rice and Barone 2000). To prevent harm, it is crucial to understand DNT potentials of chemicals, and thus testing is necessary. Therefore, we established and characterized this three-dimensional human neurosphere system that imitates the basic processes of brain development—proliferation, differentiation, and migration [Figure 2; see also Supplemental Material, Figures 1 and 2 (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)].

Individual spheres in single wells of a 96-well plate proliferated over time, and FACS analyses of propidium iodine–stained neurosphere single-cell suspensions revealed that approximately 2.72% of sphere cells went through S-phase of the cell cycle, confirming their proliferative capacity (Figure 3E,F). This is in agreement with Reynold and Rietze (2005), who found 2.4% of human neurosphere cells capable of proliferation as assessed by a single-cell clonogenic assay.

To illuminate the inside of the "black-box" neurosphere, we immunocytochemically stained proliferating spheres. Microscopic analyses illustrate a zonal distribution of nestin$^+$ hNPCs in the periphery and later GFAP$^+$ and β(III)tubulin$^+$ astrocytes and neurons in the center of the sphere (Figure 1). These findings are similar to data reported for murine neurospheres (Campos et al. 2004) and might be caused by a growth factor gradient from the sphere periphery to its inside. One could speculate that this zonal distribution resembles an "outside-in" brain structure, with nestin$^+$ cells representing the proliferative zone of the brain, which is in proximity to the growth-factor–containing liquor of the ventricles, and the GFAP$^+$ and β(III)tubulin$^+$ cells in the center of the sphere resembling superficial regions of the cortex (Campos et al. 2004). Whether the growth factor gradient is in fact responsible for zonal dissemination within a neurosphere is a subject for future investigations.

Growth factor withdrawal and presence of a poly-D-lysine/laminin matrix initiate cellular migration out of the sphere (Moors et al. 2007). Observation of initial migration over 24 hr by real-time phase-contrast microscopy illustrates that radial as well as tangential migration happens during this time [see Supplemental Material, Figure 2 (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)]. The cues causing cells to connect, disconnect, and move forward, backward, and even tangentially *in vitro* are so far unknown. Directed migration *in vivo* is motivated by chemical gradients of, for example, Netrin1/UNC6, semaphorins, or the reelin/dab1 pathway (Hatten 2002). Although some of these

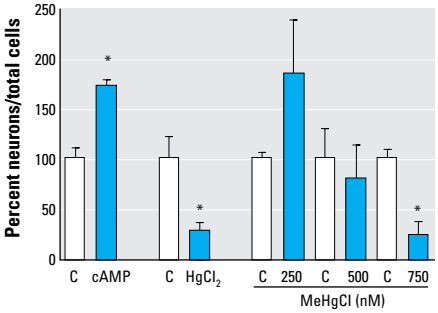

Figure 4. Chemicals disturb neurosphere differentiation. C, control. β(III)tubulin$^+$ cells/total nuclei were counted after exposing differentiating neurospheres to chemicals for 48 hr. Data are mean ± SD of at least three independent experiments (3–5 spheres per experiment).

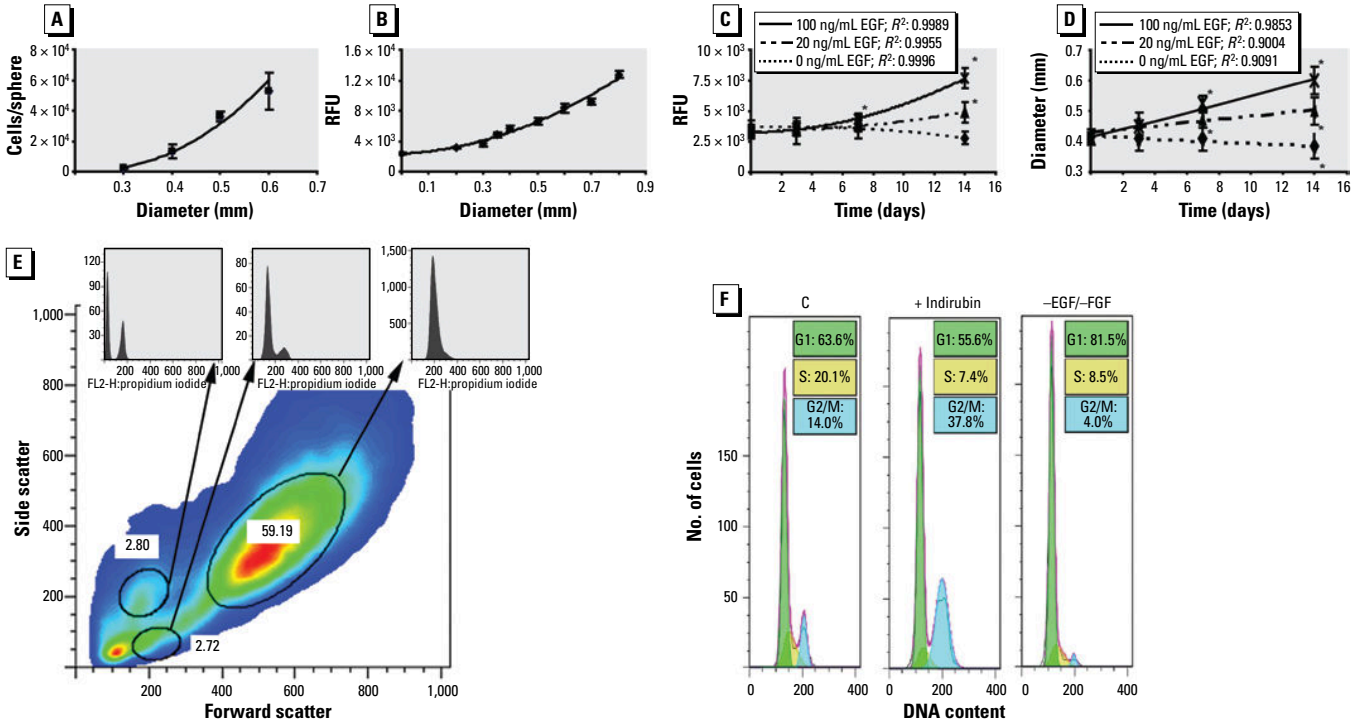

Figure 3. Assessment of neurosphere proliferation. RFU, relative fluorescence units. (*A*) Correlation between sphere diameter and number of cells/sphere (R^2 = 0.9926). (*B*) Correlation between sphere size and metabolic activity; reductase activity was measured with the CellTiter-Blue assay (R^2 = 0.983). (*C*) Measurement of sphere proliferation by assessing metabolic activity repetitively over time. (*D*) Assessment of proliferation in the same spheres shown in (*C*) by measuring sphere diameter over time. Results in (*C*) and (*D*) are typical representatives of three independent experiments at each time point shown as mean ± SD of 3–6 individual spheres. (*E*) FACS analysis of dissociated, fixed, and propidium iodine–stained neurospheres. The circled regions depict subpopulations; one contained proliferating cells (see second DNA histogram). (*F*) DNA content histograms of the proliferating cell population. The control histogram corresponds to cells cultured with EGF and FGF. A G_2/M or G_1 arrest was induced with indirubin (28 hr) or by withdrawal growth factor (96 hr).
*$p \le 0.05$.

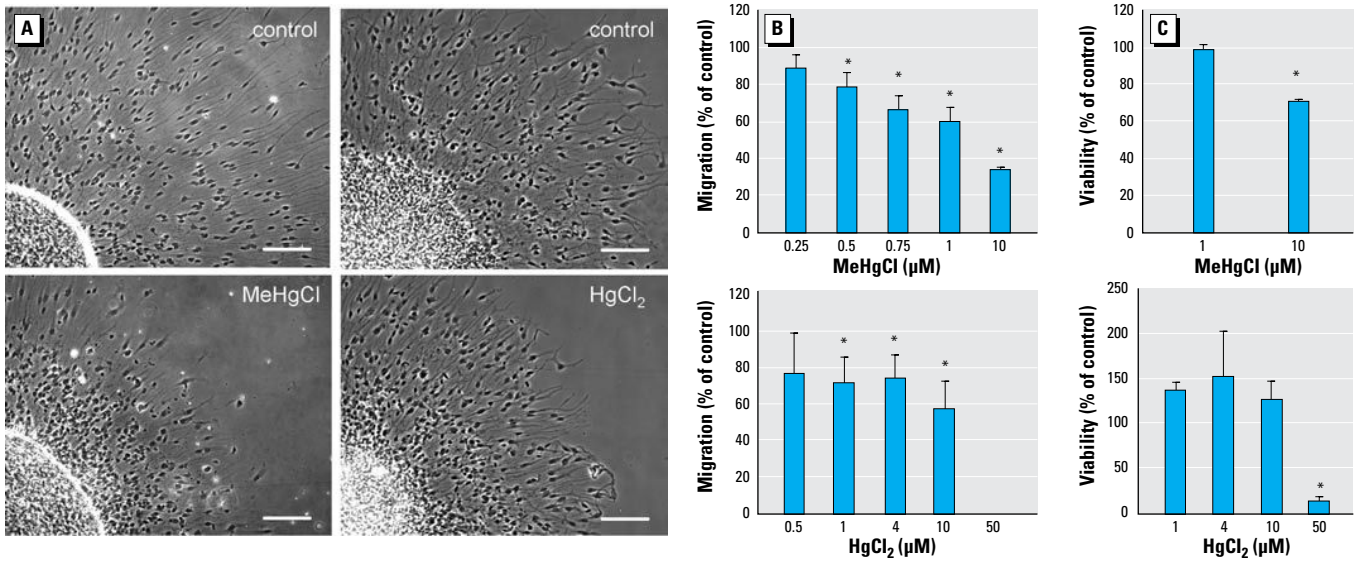

Figure 5. Mercury inhibits neurosphere migration. Phase-contrast images (*A*) and the respective quantifications (*B*) of cell migration (migration distance measured with an object micrometer from the edge of the sphere to the farthest outgrowth). In (*A*), bars = 100 μm. (*C*) Cell viability as assessed with the CellTiter-Blue assay. In (*B*) and (*C*), data are mean ± SD of at least three independent experiments (3–5 spheres/experiment).
*$p < 0.05$.

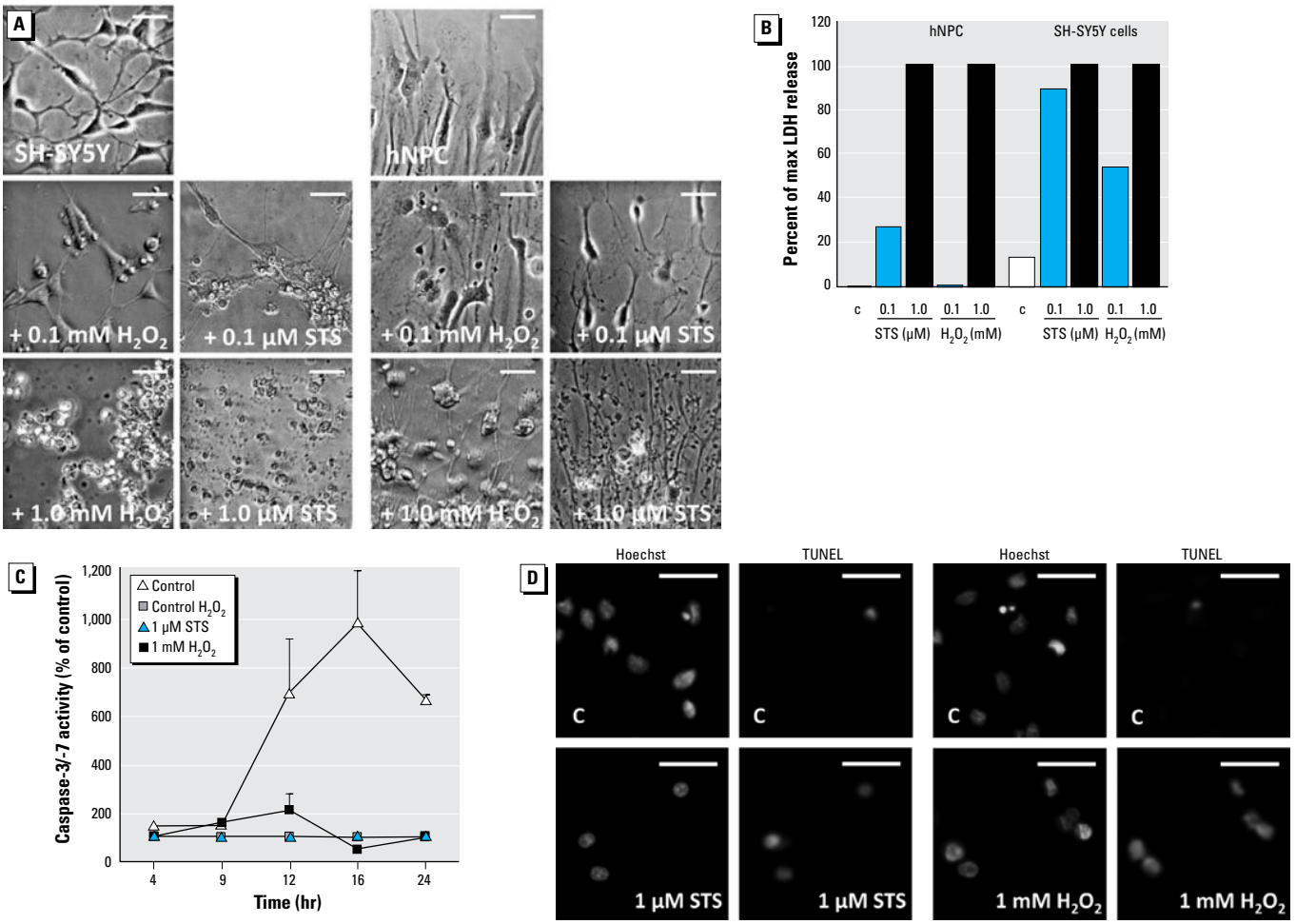

Figure 6. STS but not H_2O_2 induces caspase-dependent apoptosis. Abbreviations: C, control; max, maximum; STS, staurosporine. (*A*) Phase-contrast images and (*B*) corresponding LDH release of SH-SY5Y cells and hNPC after 24 hr of STS or H_2O_2 treatment. (*C*) Kinetic analyses of caspase-3/7 activity after STS or H_2O_2 treatment; values are typical representatives of two independent experiments (three spheres/experiment). (*D*) Cells showing positive TUNEL staining after STS or H_2O_2 exposure; nuclei are visualized with Hoechst. In (*A*) and (*D*), images are representative of two independent experiments; bars = 30 μm.

gene products, such as different semaphorins, are expressed in our neurospheres (Moors M, Fritsche E, unpublished data), whether such attractants or repellants are responsible for the directed migration we observe *in vitro* has to be further investigated. Nevertheless, the distance that differentiating hNPCs migrate over time is highly robust and reproducible. Migration speed is independent of neurosphere size and does not differ between three independent donors tested so far [gestational weeks 16–19; see Supplemental Material, Figure 1 (available at http://www.ehponline.org/members/2009/0800207/suppl.pdf)]. A faster migration speed (1 mm/24 hr) was reported for neurospheres that were prepared from postnatal brain cortices of premature infants (gestational weeks 23–25; Flanagan et al. 2006). This difference might be due to distinct culture conditions or ages of individuals.

During cellular outgrowth, hNPCs differentiate into GFAP$^+$, O4$^+$, and β(III)tubulin$^+$ glial- and neuronal-like cells while losing nestin staining (Figure 2A). The ratio of approximately 10% neuronal and 90% glial cells that we counted after 2 days of differentiation (Moors et al. 2007) resembles the physiologic distribution of brain cells in humans (Baumann and Pham-Dinh 2001). Furthermore, we found O4$^+$ oligodendrocyte precursor cells in the migration area that increase in number and degree of morphologic maturation over time (Figure 2) (Baumann and Pham-Dinh 2001). These differentiation results point to culture maturation.

For the development of *in vitro* assays that identify chemicals with DNT potential, we employed Hg, which is a human developmental neurotoxicant (Grandjean and Landrigan 2006). Prenatal Hg poisoning causes developmental delays, mental retardation, and adverse effects on memory and motor skills in children (Sanfeliu et al. 2003; Schettler 2001). Neuropathologic examinations revealed microcephaly and global brain disorganization resulting from disturbances in cell migration and division (Clarkson 2002; Schettler 2001). Moreover, postmortem brains had a decreased number of nerve cells (Castoldi et al. 2001; Choi 1989). We mimicked these effects *in vitro* by treating neurospheres with organic Hg and inorganic Hg, which were identified in human brain sections (Clarkson 2002). Hg decreased the migration distance (Figure 5A,B) and increased the glial cell/neuron ratio (Figure 4). We observed these effects at noncytotoxic concentrations, pointing to a target-cell–specific effect (Figure 5C). How do these findings correspond to Hg exposures in humans? From an Hg poisoning incident in Iraq, a lowest observed adverse effect level (LOAEL) for brain MeHg content in intoxicated mothers was calculated to be 800 ng/g, a level that caused neurologic symptoms in children (Clarkson 1993). Considering the measurements of Lewandowski et al. (2003), who determined cellular *in vitro* concentrations relative to corresponding medium Hg concentrations, this LOAEL is equivalent to an *in vitro* medium concentration of approximately 266 nM. MeHg accumulation in the fetus is higher than in adult organs, implying that the LOAEL is under- rather than overestimated. *In vitro* exposure of rodent neural stem cells to $HgCl_2$ (7–18 μM) or MeHg (2.5–5 nM) also resulted in reduced neuronal differentiation (Cedrola et al. 2003; Tamm et al. 2006). Although the sensitivity toward inorganic Hg was similar in human compared with mouse spheres (Cedrola et al. 2003), rodent stem cells treated for 7 days were more sensitive toward organic Hg than were human cultures treated for 2 days (Tamm et al. 2006). Although both mercuric compounds exert adverse effects by binding to sulfhydryl groups of proteins (Clarkson 1972), one further mode of action of organic Hg is the induction of oxidative stress (Sarafian and Verity 1991; Yee and Choi 1994). Antioxidant defenses are low in human embryonic brains and evolve during development (Buonocore et al. 2001). Furthermore, there might also be species differences in defense capacities (Knobloch et al. 2008). Thus, the differences between our and previously published results for MeHg could be due to the age of cultures (stem vs. fetal cells), species differences, or varying exposure times.

In contrast to Hg, cAMP, a well-described compound for inducing neuronal differentiation (Deng et al. 2001), caused an increased number of β(III)tubulin$^+$ cells in differentiated hNPCs (Figure 4A), demonstrating the dynamic ability of the cell system.

Deregulation of apoptosis results in developmental brain pathology or neurodegenerative diseases (Rodier 1995). Furthermore, oxidative stress induces apoptosis in many different cells types. Therefore, we attempted to trigger ROS-induced programmed cell death in hNPC cells. Although staurosporine induced caspase-dependent apoptosis, 1 mM H_2O_2 induced TUNEL$^+$ apoptotic cells without caspase-3/-7 activation, indicating that neurospheres undergo caspase-independent apoptosis (Figure 6). This is in concert with studies in primary rat cerebellar granule cells, which also responded with caspase-independent apoptosis to H_2O_2 (Dare et al. 2001). Furthermore, comparison of LDH activity of human hNPCs with the human neuroblastoma SH-SY5Y tumor cell line suggests that hNPCs are less sensitive toward oxidative stress than are SH-SY5Y tumor cells. These data also support observations that cancer cells are more susceptible to various stressors than are normal cells (Aykin-Burns et al. 2008; Hileman et al. 2004). Besides inducing apoptosis, preconditioning of mouse NPCs with a low concentration of H_2O_2 (5 μM) is cytoprotective (Sharma et al. 2008). Whether this is also true for hNPCs or even tumor cell lines needs to be investigated.

In summary, we have shown that *a*) proliferation, migration, differentiation, and apoptosis of human neurospheres can be quantified; *b*) *in vivo* effects of the developmental neurotoxicant Hg are imitated *in vitro*; and *c*) the methods applied are suitable for medium-throughput screening. Thus, our three-dimensional neurospheres offer a new, human, system-based method for DNT hazard identification. However, their applicability is limited to basic processes of brain development, because they do not resemble complex higher brain structure development such as formation of cortical layers. Moreover, they are limited in their potential to perform drug metabolism, as is fetal tissue *in vivo*. For including "maternal metabolism" in the *in vitro* system, strategies such as incubation with S9 mixes or hepatocyte coculture have to be established.

In the future, more chemicals known to cause DNT will be tested for their potential to interfere with human neurosphere performance to develop this method into a validation process and make it applicable for testing needs.

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Gassmann K, Abel J, Bothe H, Haarmann-Stemmann T, Merk HF, Quasthoff KN, Rockel TD, Schreiber T, Fritsche E

Environmental Health Perspectives [eingereicht am 07.10.2009; in Revision]

Die Struktur des AhR ist evolutionär stark konserviert und Homologe zum Säuger-AhR konnten bereits in verschiedenen Invertebraten und Fischen nachgewiesen werden. Es zeigte sich, dass ein Fehlen oder der Funktionsverlust des AhR dort zu Entwicklungsdefekten besonders innerhalb des neuralen Systems führt. Die *in utero* Exposition mit typischen AhR-Liganden wie PAHs und Dioxinen steht auch beim Menschen in Verdacht entwicklungsneurotoxische Effekte auszulösen. Mögliche Expositionsquellen sind dabei besonders das Rauchen während der Schwangerschaft und der Konsum von kontaminierter Nahrung. Ob aber auch beim Säuger diese Effekte über eine Aktivierung des AhR vermittelt werden, ist bisher kaum untersucht. Das Ziel der vorliegenden Publikation war daher die Wirkungen von bekannten synthetischen AhR-Liganden auf die humane und murine neurale Entwicklung anhand eines auf neuralen Progenitorzellen (NPC) basierenden *in vitro* Testmodells zu untersuchen und zu vergleichen.
Die Exposition mit klassischen AhR-Agonisten 3-Methylcholanthren (3-MC) und Benzo(a)pyren (B(a)p) und dem AhR-Antagonisten 3-Methoxy-4-nitroflavon (MNF) ergab, dass sowohl AhR-Agonisten als auch Antagonisten keinen Einfluss auf Viabilität, Proliferation und Migration von humanen NPCs haben, aber die Aktivierung des AhR im murinen NPCs zur einer Reduzierung der Migration und eine Antagonisierung mit MNF zu einer kompletten Inhibierung der Proliferation führt. Weiterführende Untersuchungen zeigten, dass eine deutlich niedrigere Expression und fehlende Aktivierbarkeit des AhR in hNPCs diesen spezies-spezifischen Effekten zugrunde liegt. Die Daten der vorliegenden Publikation weisen somit darauf hin, dass der Mensch in diesem Entwicklungsstadium (GW 16) deutlich unempfindlicher in Bezug auf AhR-vermittelte Wirkungen zu sein scheint. Dieser Punkt hat besondere Wichtigkeit für die Regulation der entsprechenden Substanzen, da die Grenzwertsetzung bisher nach wie vor auf der Extrapolation von Labornagerdaten beruht.

Die Verfasserin der Dissertation hat alle der Publikation zugrunde liegenden Experimente geplant und bis auf die quantitativen RT-PCRs der hNPCs auch selbst durchgeführt. Die Maus-Neurosphären wurden freundlicherweise von Timm Schreiber und Kim Quasthoff zur Verfügung gestellt.

ehp ENVIRONMENTAL HEALTH PERSPECTIVES

Species-specific AhR-deficiency protects human neural progenitor cells against developmental neurotoxicity of PAHs

Journal:	*Environmental Health Perspectives*
Manuscript ID:	Draft
Manuscript Type:	Research Article
Date Submitted by the Author:	
Complete List of Authors:	Gassmann, Kathrin; Institut für umweltmedizinische Forschung, Toxicology Abel, Josef; Institut für umweltmedizinische Forschung, Toxicology Bothe, Hanno; Institut für umweltmedizinische Forschung, Toxicology Haarmann-Stemmann, Thomas; Institut für umweltmedizinische Forschung, Toxicology Merk, Hans; RWTH Aachen, Universitaetshautklinik Quasthoff, Kim; Institut für umweltmedizinische Forschung, Toxicology Rockel, Thomas; IUF, Toxicology Schreiber, Timm; Institut für umweltmedizinische Forschung, Toxicology Fritsche, Ellen; Institut für umweltmedizinische Forschung
Keywords:	DNT, neurospheres, PAH, AhR, species difference

Species-specific AhR-deficiency protects human neural progenitor cells against developmental neurotoxicity of PAHs

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Running title

Species-specific AhR-mediated DNT

Keywords

DNT, neurospheres, PAH, AhR, species difference

Acknowledgment and Grant information:

This work was supported by the German Research Foundation (GRK1427) and by the German Ministry for the Environment.

Abbreviations

AhR	arylhydrocarbon receptor
ARNT	AhR nuclear translocator
B(a)P	benzo(a)pyrene
bHLH/PAS	basic Helix-Loop-Helix/Per-ARNT-Sim
CNS	central nervous system
CYP	cytochrome P450
DEPC	diethylpyrocarbonate
DNT	developmental neurotoxicity
DREs	dioxin responsive elements
EGF	epidermal growth factor
ERK	extracellular-signal regulated kinase
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
rhFGF	recombinant human fibroblast growth factor
IQ	intelligence quotient

LDH	lactate dehydrogenase
3-MC	3-methylcholanthrene
MEFs	mouse embryonic fibroblasts
MeHgCl	methylmercury chloride
MNF	3'methoxy-4'nitroflavone
NPCs	neural progenitor cells
PAHs	polycyclic aromatic hydrocarbons
PCBs	polchlorinated biphenyls
PDL	poly-D-lysine
PKB/Akt	protein kinase B
POPs	persistent organic pollutants
SEM	standard error of the mean
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin

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Abstract

Background: Due to their lipophilicity, persistent organic pollutants (POPs) cross the human placenta, possibly affecting central nervous system (CNS) development. The majority of POPs are known AhR ligands and activators of AhR signaling. Therefore, it has been proposed that AhR activation causes developmental neurotoxicity (DNT).

Objective: Whether AhR-activation is the underlying mechanism for reported DNT of POPs in humans is not known. Thus, effects of AhR ligands on basic processes of brain development were studied in two comparative *in vitro* systems.

Methods: We employed neurosphere cultures based on human and mouse neural progenitor cells (hNPCs & mNPCs) and studied the effects of different AhR agonists and an antagonist on neurosphere development. Moreover, we analyzed expression of genes invoved in Ahr signaling.

Results: In contrast to mNPCs, hNPCs are insensitive to AhR agonism or antagonism. While AhR modulation attenuates mNPC proliferation and migration, hNPCs remain unaffected. We also show that species-specific differences result from non-functional AhR-signaling in human neurospheres.

Conclusion: In summary, we show that in contrast to mouse, human neurospheres are protected against PAH-induced DNT due to absence of AhR which should be taken into account for risk assessment of POPs.

Introduction

Persistent Organic Pollutants (POPs) bioaccumulate through the food chain and pose a risk of causing adverse effects to human health and the environment. Main substance classes are polycyclic aromatic hydrocarbons (PAHs), like 3-methylcholanthrene (3-MC) and benzo(a)pyrene (B(a)P), dioxins like 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated biphenyls (PCBs). Due to their lipophilicity, POPs cross the human placenta exposing the fetus to the contaminant body burden of the mother. This results in increased adverse health outcomes, possibly affecting central nervous system (CNS) development (rev. in Wormley et al. 2004). One potential source for *in utero* exposure to POPs, especially to PAHs, is maternal cigarette smoking during pregnancy (Polanska et al. 2009; Rodgman et al. 2000). It is known that infants of mothers who smoke during pregnancy are at an increased risk to be born with a smaller head (Kallen 2000; Lindley et al. 1999). This smaller head circumference might be associated with impaired brain development, because head growth *in utero* is a predictive factor for IQ scores, reading ability, and problem-solving skills (Frisk et al. 2002). An inverse relationship between maternal smoking during pregnancy and offspring intelligence (IQ) and cognitive ability has also been described in numerous cohort studies (Batty et al. 2006; Fried et al. 1998; Olds et al. 1994). Additionally, behavioral problems and psychiatric disorders in offspring have been associated with smoking during pregnancy (Thapar et al. 2003; Weissman et al. 1999;Fergusson et al. 1998). Next to smoking, *in utero* exposure to PCBs and/or dioxins results in cognitive deficits in children (rev. in Grandjean and Landrigan 2006; Jacobson and Jacobson 1997; Patandin et al. 1999). These alterations are thought to involve the POP-activated arylhydrocarbon receptor (AhR), an evolutionary highly conserved member of the basic Helix-Loop-Helix/Per-ARNT-Sim (bHLH/PAS) family of transcription factors. Ligand binding to this cytosolic recptor induces nuclear translocation (Gasiewicz and Bauman 1987) and heterodimerization with another bHLH/PAS protein,

ARNT (AhR nuclear translocator; (Reyes et al. 1992)). The resulting complex recognizes and binds specific DNA sequences, i.e. dioxin responsive elements (DREs), within gene promoter regions and modulates subsequent transcription of AhR-dependent genes (Fujisawa-Sehara et al. 1987). Most of the above discussed substance classes like PAHs and dioxins are known AhR ligands and activators of AhR signaling. It has therefore been proposed that AhR activation causes developmental neurotoxicity (DNT). This hypothesis is supported by studies in invertebrate (*C. elegans*) and vertebrate (zebrafish, chicken, rat, monkey) species where dioxins and related compounds cause morphological abnormalities of brains or deficits in cognition and/or behaviour (Henshel et al. 1997; Hill et al. 2003; rev. in Kakeyama and Tohyama 2003; Qin and Powell-Coffman 2004). Whether AhR-activation is the underlying mechanism for the reported DNT effects after POP exposure in humans is not known.

To answer this question we employed comparative *in vitro* test systems for brain development based on neurosphere cultures from human and mouse neural progenitor cells (hNPCs and mNPCs, respectively). These three dimensional cell systems mirror basic processes of fetal brain development such as proliferation, migration, differentiation and apoptosis. Moreover, they detect developmental neurotoxicants *in vitro* (Fritsche et al. 2005; Moors et al. 2007; Moors et al. 2009). We here report that in contrast to mNPCs, hNPCs are insensitive to AhR agonism or antagonism due to non-functional AhR-signaling in human neurospheres indicating that humans are protected towards AhR-dependent, POP-induced DNT. Knowledge about such species-specific differences is of outmost importance with regard to chemical testing and hazard assessment for humans.

Material and Methods

Chemicals

The AhR antagonist 3′methoxy-4′nitroflavone was kindly provided by G. Vielhaber (Symrise, Holzminden, Germany). Methylmercury chloride (MeHgCl) was obtained from Riedel-de Haën (Seelze, Germany). All additional chemicals used (unless otherwise noted) were purchased from Sigma–Aldrich (Munich, Germany) and were of the highest purity available.

Cell culture

HNPCs used in this study were purchased from Lonza (Verviers SPRL, Belgium). For mouse neurosphere culture, brains of C57/BL6 mice (Charles River) were removed at embryonic day (E) 15.5-16.5 and transferred to phosphate buffered saline (PBS). Aage of the embryos was determined according to the staging criteria of Theiler, in which E16 correspond to Theiler stage 24 (Bard et al. 1998). Brains of three embryos were dissected, transferred to DMEM and mechanically dissociated. Trypsin/EDTA solution was added and incubated for 30 min at 37°C in a humidified atmosphere. Afterwards, the tissue suspension was triturated to obtain a single cell suspension which was centrifuged with 800 rpm for 5 min. Pellets were resuspended and plated in 10 cm petridishes. Presented data is derived from four independent preparations.

Both, hNPCs and mNPCs were cultured in proliferation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/ml EGF (Biosource, Karlsruhe, Germany), 20 ng/ml rhFGF (R&D Systems, Wiesbaden-Nordenstadt, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin) in a humidified 92.5% air/7.5% CO2 incubator at 37 °C in suspension culture. Differentiation was initiated by growth factor withdrawal in differentiation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with N2 (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin) and plating onto poly-D-lysine (PDL)/laminin coated chamber slides.

Cell Viability

Cell viabiltiy was measured using a lactate-dehydrogenase (LDH) assay (CytoTox-One, Promega) according to the manufacturer's instructions. Briefly, supernatants of treated cells from the migration, mRNA-expression and proliferation assessments were collected at the respective timepoints and incubated 2:1 with the CytoTox-One reagent for 4 h prior to detection of fluorescence (Ex 540 nm/Em 590 nm). Complete lysis of cells with the included lysis buffer for 2 h at room temperature serves as a positive control.

Proliferation Assays, Cell Cycle Analyses and Migration Assay

Proliferation was assessed with a combination of CellTiter-Blue Assay (Promega, Madinson, USA), which measures mitochondrial reductase activity, and microscopical determination of sphere diameter as described previously (Moors et al. 2009). Ccell cycle was analysed by FACS. Therefore, neurospheres were exposed to the different chemicals for 48 h, singularized with accutase, fixed with paraformaldehyde and stained with propidium iodine (Moors et al 2009). Migration distance was measured microscopically after 48 hours as previously described (Moors et al. 2007; Moors et al. 2009).

Quantitative Real-Time PCR

Neurospheres were treated under differentiating conditions with 10 µM 3-MC or 0.1 % DMSO as solvent control. After indicated timepoints, RNA was prepared with the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Real-time reverse-transcriptase polymerase chain reaction (rtRT-PCR) was performed using the LightCycler instrumentation (Roche, Mannheim, Germany) with the QuantiTect SYBR green PCR Master Mix (Qiagen, Hilden, Germany) as previously described (Fritsche et al. 2005). Conditions for PCR amplifications were initial 15 min at 95°C, 40 cycles of 15 sec at 94°C for denaturation, 25 sec of primer annealing, 30 sec at 72°C for

elongation, and 2 sec at 72°C for fluorescence detection. Intron-spanning primers were designed using PRIMER 3 Software to reduce genomic amplification. Primer sequences for hBeta-Actin, hAhR, hAhRR, hCYP1A1, hCYP1B1 were described by Fritsche et al. (Fritsche et al. 2005). Other primer sequences are: **hARNT**: (F) CCCTAGTCTCACCAATCGTGGAT (R) GTAGCTGTTGCTCTGATCTCCCAG **hC-Myc**: (F) ACCACCAGCAGCGACTCTGA (R) TCCAGCAGAAGGTGATCCAGACT (56°C annealing), **msBeta-Actin**: (F) CTACAATGAGCTGCGTGTGG (R) TAGCTCTTCTCCAGGGAGGA (60°C annealing), **msAhR**: (F) GACAGTTTTCCGGCTTCTTG (R) CGCTTCTGT AAATGCTCTCGT (60°C annealing), **msARNT**: (F) TGCCTCATCTGGTACTGCTG (R) GAACATGCTGCTCACTGGAA (58°C annealing), **msAhRR**: (F) GTTGGATCCTGTAGGGAGCA (R) AGACCAGAGGCTCACGCTTA (60°C annealing), **msCYP1A1**: (F) GGCCACTTTGACCCTTACAA (R) CAGGTAACGGAGGACAGGAA (54°C annealing), **msCYP1B1**: (F) ACATGAGTTTCAGTTATGGCC (R) TTCCATTCACTGCTGAGAGC (58°C annealing), **msC-Myc**: (F) TGTCCATTCAAGCAGACG (R) GCATTTTAATTCCAGCGCATAG (54°C annealing). Expression levels were normalized to the expression of beta-actin. Gene expression was evaluated using the Ct value from each sample. The method of calculation is based on the method of the ΔΔCt (Livak and Schmittgen 2001). For determining absolute copy numbers product-specific standards amplified from cDNA were used to generate standard curves. A target gene was considered to be quantifiable if the ratio copy number target gene/copy number beta-actin x1000 exceeded 0.001.

Statistics

All results are mean and SEM of at least three independent experiments. We used analysis of variance combined with the Dunnetts post hoc test for multifactor analyses (concentration

effects), and the Student's *t*-test for two-group comparisons (treatment vs. control) Significance value was set at $p< 0.05$.

Results

AhR agonism or antagonism does not cause cytotoxicity in hNPCs and mNPCs.

AhR agonists 3-MC and B(a)P or AhR antagonist MNF does not induce cytotoxicity (LDH release) of human or mouse neurospheres (Suppl. Material).

Proliferation of mNPC, but not hNPCs is inhibited by AhR blockage.

Figure 1A and B show quantifications of the proliferation assays (sphere diameter and Celltiter-Blue Assay, respectively) of human neurospheres after exposure to 1 and 10 µM 3-MC or 1 µM MNF. Both assays showed no significant disparity between the different exposures after 7 and 14 days. After 7 and 14 days, all human neurospheres except the ones without growth factors had grown from a diameter of 425 µm ± 7 µm to a diameter of 632 µm ± 23 µm and 824 µm ± 23 µm, respectively. These results were confirmed by FACS analyses for DNA content using propidium iodine staining (Figure 1C). Independent of exposure conditions, the mean number of cells was in G0/G1-phase (53.5% ± 3.4%), while 28% ± 1.8% were in S-phase, and 18.4% ± 2.8% cells were in G2/M-phase after 48h of exposure.

In contrast to the human data, there was a significant inhibition of mNPC proliferation after 7 days of 1 µM MNF exposure (Figure 1D and E). While control and 3-MC exposed spheres grew from a diameter of 406 µm ± 3 µm to a diameter of 495 µm ± 12 µm, MNF-exposed spheres remained at 405 µm ± 78 µm. These results were confirmed by FACS analyses for DNA content using propidium iodine staining (Figure 1F). In the solvent control, 56.1% ± 4.6% of cells were in G0/G1-phase, 38.1% ± 4.3% were in S-phase, and 5.8% ± 0.4% cells were in G2/M-phase after 48h of exposure. 3-MC treated cells showed a similar distribution.

However, MNF treatment reduced the number of cells in G2/M-phase to 2.0% ± 0.4% while the distribution of cells in G0/G1- and S-phase remained relatively constant at 51.8% ± 0.2% and 46.3% ± 0.4%, respectively.

Neural cell migration is affected by AhR stimulation in mouse, but not human neurospheres.

Next, we investigated if 3-MC, B(a)P or MNF influence human and mouse NPC migration. Therefore, neurospheres were exposed to 1 and 10 µM 3-MC, 10 µM B(a)P or 1 and 0.1 µM MNF while differentiating for 48h. 0.75 µM MeHgCl served as a positive control for inhibition of migration. Independent of exposures, all human neurospheres adhered to the PDL/laminin matrix and migrated radially out of the sphere with an average migration distance of 764 µm ± 63 µm after 48h (Figure 2A & C).

In contrast to the results in hNPCs, AhR activation by 3-MC and B(a)P reduced mNPC migration distance 16% ± 5% (1 µM 3-MC), 21% ± 13% (10 µM 3-MC) and 32% ± 10% (10 µM B(a)P) compared to solvent controls, while MNF had no effects (Figure 2B & D).

AhR-dependent gene transcription is only inducible in mNPCs, but not in hNPCs due to lacking AhR and ARNT transcripts in human cells.

Because 3-MC, B(a)P and MNF did not influence hNPC viability, proliferation or migration, but modulated proliferation or migration of mNPCs, we determined AhR and ARNT mRNA expression under proliferating and differentiating conditions in hNPCs and mNPCs. AhR and ARNT mRNAs were expressed in human and mouse NPCs independent of culture condition, but expression of the AhR target genes AhRR, CYP1B1 and c-Myc was not inducible by 10 µM 3-MC after 6h, 12h, 24h and 48h of differentiation in hNPCs (Figure 3A). The expression level of CYP1A1 was undetectable in hNPCs.

In mNPCs, cyp1a1 and cyp1b1 were significantly induced by 10 µM 3-MC after 6h to 6.6 ± 1.7- and 2.5 ± 0.25-fold of controls, respectively, while AhRR and c-Myc expression remained unaffected (Figure 3B).

Comparison of mRNA expression levels between human and mouse NPCs showed that genes of AhR signaling and AhR gene battery were generally expressed in higher copy numbers in mNPCs than in hNPCs (Figure 3C). AhR mRNA level was 8.2-fold higher for proliferating and 102.2-fold higher for differentiating spheres (mNPCs/hNPCs), ARNT mRNA level was 55.3- fold higher for proliferating and 77.1-fold higher for differentiating spheres and AhRR mRNA expression was 84.2- fold higher for proliferating and 638.9-fold higher for differentiating spheres. Interspecies-difference was highest for CYP1B1 mRNA expression with a factor of 1.6×10^4 for proliferating and 1.5×10^4 for differentiating spheres. These results give rise to the hypothesis that greater expression levels of AhR signaling pathway gene products cause toxicity of the AhR agonists 3-MC and B(a)P and the AhR antagonist MNF in mNPCs.

Discussion

The development of cell-based, non-animal testing strategies for hazard assessment of chemicals is currently one of the most important tasks in toxicological research. In this regard, it is most important to choose appropriate model systems which are truly predictive for humans (National Research Council. 2007;Krewski et al. 2009). Human tumor cell lines which are easily accessible in large quantities bear the restriction that they do not represent cellular metabolism and signal transduction of normal cells. On the contrary, primary cells are often obtained as *ex vivo* cultures from rodents. Such primary cultures are regarded as superior over tumor-derived cells. However, species-specific differences limit their application. One example of how rodent primary cells can indeed misclassify hazards for

humans is given in this study, where mouse-derived primary cells were shown to be more susceptible towards AhR modulation than their human counterparts. With regard to chemical testing, it is fundamental to be aware of such differences to not over- or underestimate hazards that chemicals pose to humans and thereby protect man and allow industry production and development of chemicals at the same time.

Specifically, in this study we discovered that proliferation of hNPCs was not affected by AhR agonists or antagonist (Figure 1A-C), while proliferation of mNPCs was completely blocked by AhR antagonism (Figure 1D-F). Our results of the proliferation analysis in hNPCs are in contrast to findings obtained in human liver or neuroblastoma tumor cells which show that exogenous AhR activation by TCDD or other AhR ligands inhibits cell proliferation and induces cell cycle arrest (Jin et al. 2004; Marlowe and Puga 2005). On the contrary, high AhR content promotes proliferation in a human MCF breast cancer cell line which was blocked by the AhR antagonist MNF or selective AhR knockdown via siRNA (Wong et al. 2009). In human umbilical vascular endothelial cells though, AhR activation by 3-MC also exerted antiproliferative effects as was seen in the tumor cell lines (Pang et al. 2008). These data are examples of the previously described cell type-specificity of AhR-related effects. Not only the condition normal/vs. tumor cells determines if AhR activation causes cell cycle progression or arrest, but also different cell types of the human body react differently towards AhR activation or inhibition.

In contrast to hNPCs, proliferation of mNPCs is completely blocked by MNF (Figure 1D-F). This is in agreement with antiproliferative actions of flavone and alpha-naphthoflavone on the AhR-proficient murine hepatoma cell line 1c1c7 (Reiners, Jr. et al. 1999). The assumption that the AhR is the probable target for these antiproliferative effects is supported by the observation that the AhR-defective variant of the mouse hepatoma Hepa 1c1c7 cell line exhibits a prolonged doubling time compared with its wild-type counterpart (Ma and

Whitlock, Jr. 1996). Moreover, mouse embryonic fibroblasts (MEFs) from AhR knockout mice also grow more slowly than wild-type cells (Elizondo et al. 2000). On the contrary, AhR knockout animals show accelerated proliferation in different organs like skin, hair follicles and liver blood vessels. Moreover, these animals have a higher age-dependent incidence of adenocarcinomas of liver and lung (rev. in Marlowe and Puga 2005). Thus, as in humans, AhR effects on proliferation are also cell type-dependent in rodents and, as our data indicate, cell type-specificity is not consistent throughout species.

Besides progenitor cell proliferation, migration is another essential process in brain development. To address the role of the AhR in this process, we employed the neurosphere migration assay where the distance that is covered by cells migrating radially out of the sphere is measured over time (Moors et al. 2007). Migration of hNPCs was initiated and continued for 48 h in the presence of 3-MC, B(a)P and MNF. AhR modulation did not affect hNPC migration (Figure 2A & C). These data are in contrast to the two existing publications on this topic in human tumor cells showing increased motility and migration of MCF-7 cells after TCDD and 3-MC treatment in a scratch assay (Diry et al. 2006) and in a transwell migration assay (Seifert et al. 2009) indicating that AhR-mediated effects on cell migration differ between normal and tumor cells and/or cell types.

The AhR however can be involved in neural progenitor cell migration, because we show that AhR stimulation reduces migration of mNPCs (Figure 2B & D). This is supported by an earlier *in vivo* study where prenatal exposure to the AhR ligand 7,12-dimethylbenz[a]anthracene disrupts cerebellar cytoarchitecture in rats (Kellen et al. 1976). Conversely, other rodent *in vitro* and *in vivo* studies showed opposite effects in other cell types. Immortalized mouse mammary fibroblasts from AhR-null mice had decreased migration capacities in culture and this phenotype was associated with an increased stress fiber formation and with a lower efficiency to induce lamellipodia (Mulero-Navarro et al. 2005). Signaling pathways that regulate cell migration were also inhibited. A lower activation

of focal adhesion kinase (FAK), protein kinase B/AKT (PKB/AKT), mitogen activated protein kinase ERK1 and Rac-1 was detected (Mulero-Navarro et al. 2005). That ERK-dependent pathways are also necessary for normal migration of hNPCs was recently shown by our group (Moors et al. 2007). However, the AhR does not seem to determine ERK-dependent migration in hNPCs as migration distance does not change in presence of AhR modulators (Figure 2A & C) but is impaired by ERK inhibition (Moors et al. 2007). Taken together, existing data on AhR-dependent migration implies that – as for proliferation – modulation of cell migration by AhR presence or AhR ligands differs between cell types and species.

To address the underlying reason for the observed species-specific differences in response to AhR modulation between human and mouse NPCs, we quantified copy numbers of genes which belong to the AhR machinery as well as genes which are AhR-regulated. Both, human and mouse NPCs express AhR, ARNT, AhRR, CYP1B1 and c-Myc (Figure 3A & B). However, only mNPCs express quantifiable amounts of cyp1a1 and respond to 3-MC treatment with a time-related induction of cyp1a1 and cyp1b1 mRNA (Figure 3B). Inducible cyp activity by 3-MC was also measured in rat neuronal and glial cells (Dhawan et al. 1990). Moreover, mouse cerebellar granule neuroblast cultures elicited time- and concentration-dependent increases in cyp1a1 and 1b1 mRNA and protein levels after TCDD treatment (Williamson et al. 2005) which was also true for brain cyp1a1 mRNA expression *in vivo* (Huang et al. 2000). As transcriptional induction of CYP1A1 and 1B1 by PAHs is mediated through the AhR, we suspected that the non-inducibility of AhR target genes in hNPC might be due to non-functional AhR signaling. Indeed, although hNPC AhR mRNA is detectable by rtRT-PCR, copy numbers of AhR and ARNT are close to detection limit and 8- to 100-fold lower than in mNPCs (Figure 3C). Moreover, human AhR ligand affinity is approximately 10-fold lower than C57/BL6 mouse AhR-ligand attraction (Harper et al. 1988) attributable to an amino acid substitution in the human AhR ligand-binding domain (Ema et al. 1994). This

mutation is also responsible for divergent toxic potencies of TCDD between responsive C57/BL6 and non-responsive DBA mice strains (Ema et al. 1994). The irresponsiveness of hNPCs towards AhR modulation may hence be a function of low AhR expression combined with a human low affinity receptor for ligand binding.

Species-differences similar to those reported here were observed in a comparative study of human and mouse palate organ cultures. This earlier work was driven by the fact that TCDD induces cleft palate in mouse embryos and that the risk for humans to develop such malformations upon *in utero* exposure towards TCDD or related compounds was not known. This study shows that (i) human palate organ cultures express approximately 350 times less AhR mRNA than the mouse cultures, (ii) the levels of CYP1A1 mRNA in mouse were 435-fold greater than in human and (iii) human palates required 200 times more TCDD to produce a cleft palate *in vitro* than in the respective mouse model. The authors concluded that it seems highly unlikely that human embryos are exposed to sufficient amounts of TCDD to cause interruption of palatal differentiation (Abbott et al. 1999). Our results support those findings in a different organ system, the developing brain, and indicate that studies on AhR-dependent developmental neurotoxicity in C57/BL6 mice *in vivo* overestimate the risk for disturbances of human brain development resulting from AhR activation. Our data also imply that the use of toxic equivalency factors (TEF), which correspond to the relative potency of a chemical to generate AhR-mediated effects compared to TCDD, for risk assessment of POPs is not necessarily useful.

How can the epidemiological evidence for POP-related DNT in humans be explained if the AhR is not involved in chemically-induced DNT? There are three alternative mechanisms discussed how POPs might interfere with human brain development (comprehensively rev. in Kodavanti 2005). One possibility may be POP-induced changes in neurotransmitters like dopamine or serotonine which could change among others learning and memory. As those

impacts on brain development extend beyond the basic processes of brain development investigated in this study, the basic 'neurosphere assay' (Breier et al. 2009) cannot pick up such changes. As certain neurotransmitter synthesizing enzymes are expressed in neurospheres (Fritsche and coworkers, unpublished observations), one could, however, extend future DNT testing in neurospheres for endpoints reflecting neurotransmitter synthesis. Second, PCBs were found to alter intracellular phosphokinase C (PKC) signaling and Ca^{2+} homeostasis in rodents. As PKC modulation by the inhibitor BisI or the stimulator PMA causes inhibition and stimulation of hNPC migration, respectively (Moors et al. 2007), it is unlikely that the POPs used in this study affect PKC signaling. Third, and most likely, effects on thyroid hormone (TH) balance might contribute to POP-induced DNT. Thereby, some POPs can elicit direct biological effects on target organs like the brain by interfering with cellular TH signaling *in vivo* and *in vitro* (Zoeller and Crofton 2000;Fritsche et al. 2005; Schreiber et al. 2009). As the 'neurosphere assay' is able to pick up endocrine disruption of TH signaling by PCBs and polybrominated diphenyl ethers (PBDEs) (Fritsche et al. 2005; Schreiber et al. 2009), this mechanism is hence unlikely to be involved in AhR-independent DNT of 'classical' AhR ligands like 3-MC or TCDD in humans. Moreover, chemicals exert systemic effects on TH homeostasis by directly affecting the thyroid gland and decrease the synthesis of TH, reducing blood TH levels by enhanced TH metabolism, or displacing natural ligand (T4) binding to the TH plasma transport protein transthyretin. That TCDD affects systemic TH balance also in humans was recently shown by Baccarelli et al. (2008) who found that in the highly TCDD-exposed Seveso cohort maternal exposure produces effects on neonatal thyroid function (Baccarelli et al. 2008).

In summary, we show that in contrast to mouse, human neurospheres are protected against PAH-induced DNT due to absence of AhR. An accumulating body of evidence now indicates that human AhR signaling is less operative than AhR function in most laboratory animals.

This knowledge should be taken into account for risk assessment of TCDD and related xenobiotics.

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Figure Legends

Figure 1: MNF inhibits mNPC but not hNPC proliferation. NPCs were exposed to 3-MC and MNF for 7 (mNPCs) or 14 days (hNPCs). After 7 or 14 days sphere diameters and metabolic activities of human (A & B) and mouse (D & E) neurospheres were measured. The results of the two endpoints were plotted in separate xy-diagrams and the gradient of the linear regression curve was assessed after 7 or 14 days. Data represent the mean gradients ± SEM of 4 independent experiments (5-6 spheres/exposure). Data were analyzed by One-way ANOVA and Dunnetts post test (* = $p < 0,05$ vs. PC). Cell cycle phase distributions of dissociated, fixed, and propidium iodine–stained human (D) and mouse (F) neurospheres were analyzed by FACS. Shown is the mean ± SEM of 3 independent experiments after 48h exposure to 10 µM 3-MC or 1 µM MNF. PC = 0.1% DMSO with growth factors; NC = 0.1% DMSO without growth factors. (* = $p < 0,05$ vs. PC).

Figure 2: AhR agonists shorten mNPC but not hNPC migration. Human (A & C) and mouse neurospheres (B & D) were exposed to 3-MC, B(a)P, MNF or MeHgCl during differentiation for 48h. Migration distance from the edge of the sphere to the furthest outgrowth was measured. Data represent mean ± SEM of at least 5 independent experiments (5-8 spheres/exposure). Data were analyzed by One-way ANOVA and Dunnetts post test (* = $p < 0,05$ vs. positive control). Scale bar = 500 µm

Figure 3: 3-MC induces cyp mRNA expression in mNPCs. hNPC (A) and mNPC (B) mRNA expressions for indicated genes were analysed 6, 12, 24, 48h after 10 µM 3-MC exposure. Copy numbers of the respective genes were normalized to beta-actin and are expressed as fold induction compared to vehicle control (DMSO). Shown are means ± SEM of four independent experiments. Data were analyzed by student's t-test (* = $p < 0,05$ vs.

vehicle control). (C) Comparison of human and mouse mRNA copy numbers/1000 copies of the beta-actin of proliferating and 24h differentiating NPCs. Data represents at least three independent experiments. To compare between human and mouse mRNA expression levels, the factor mouse/human is calculated (n.d. = not determinable).

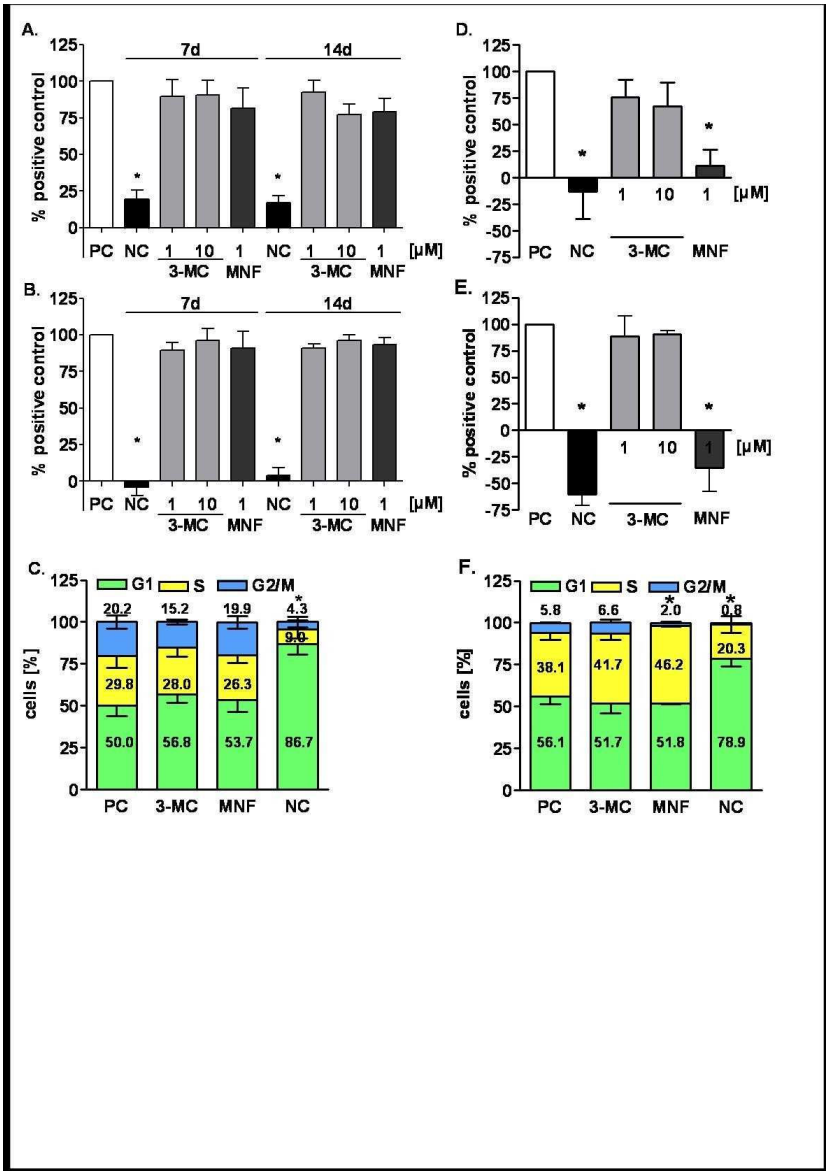

Figure 1
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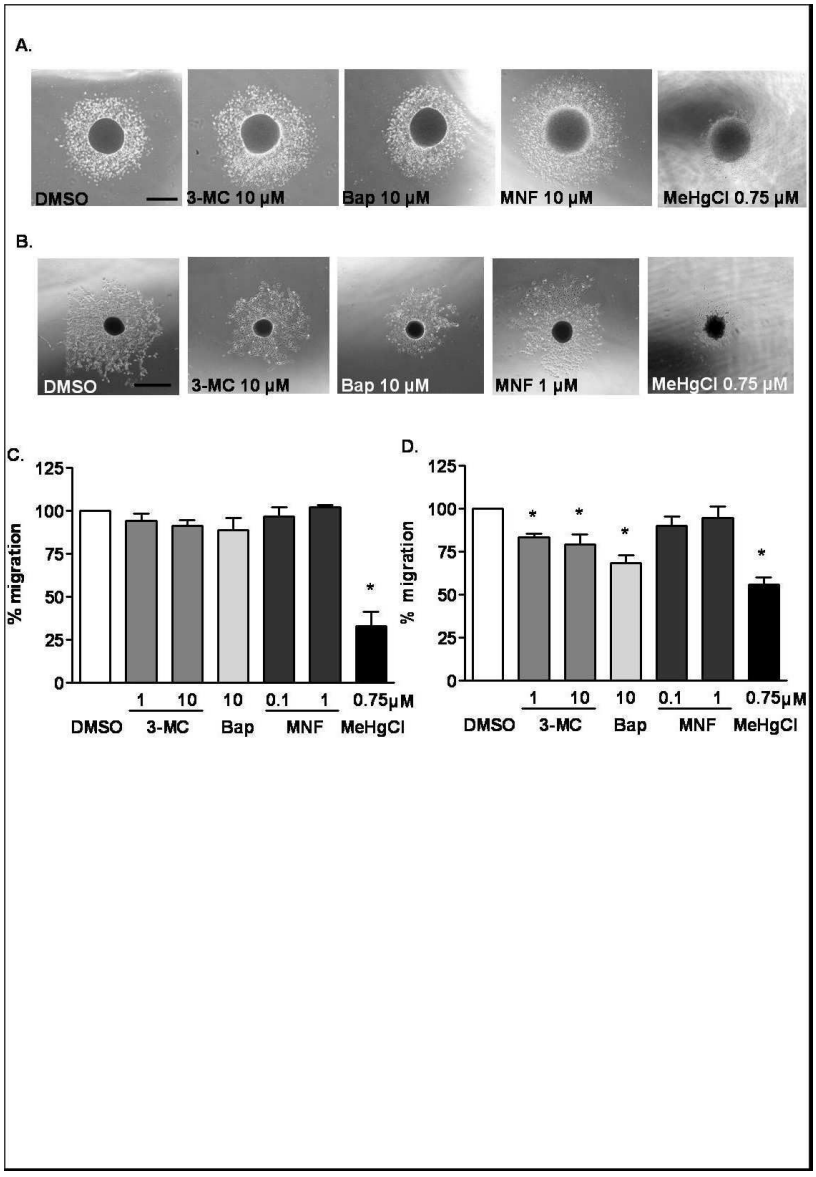
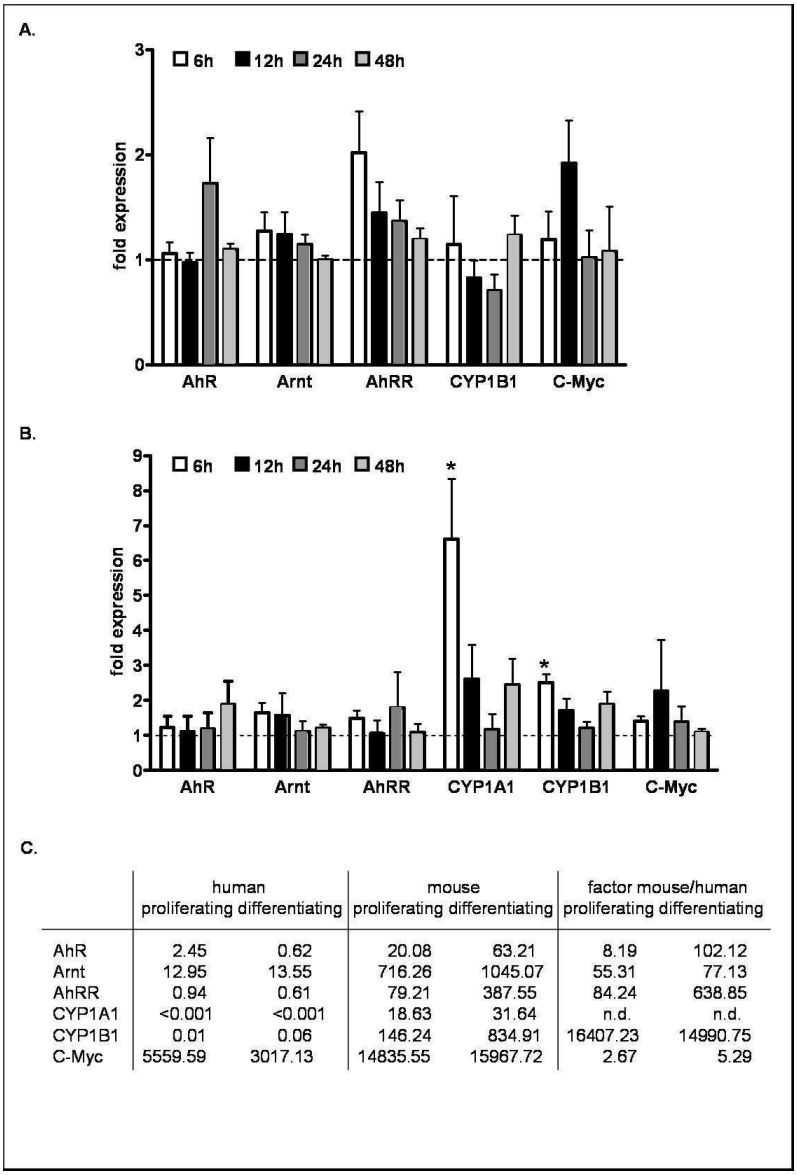


Figure 2
95x137mm (300 x 300 DPI)

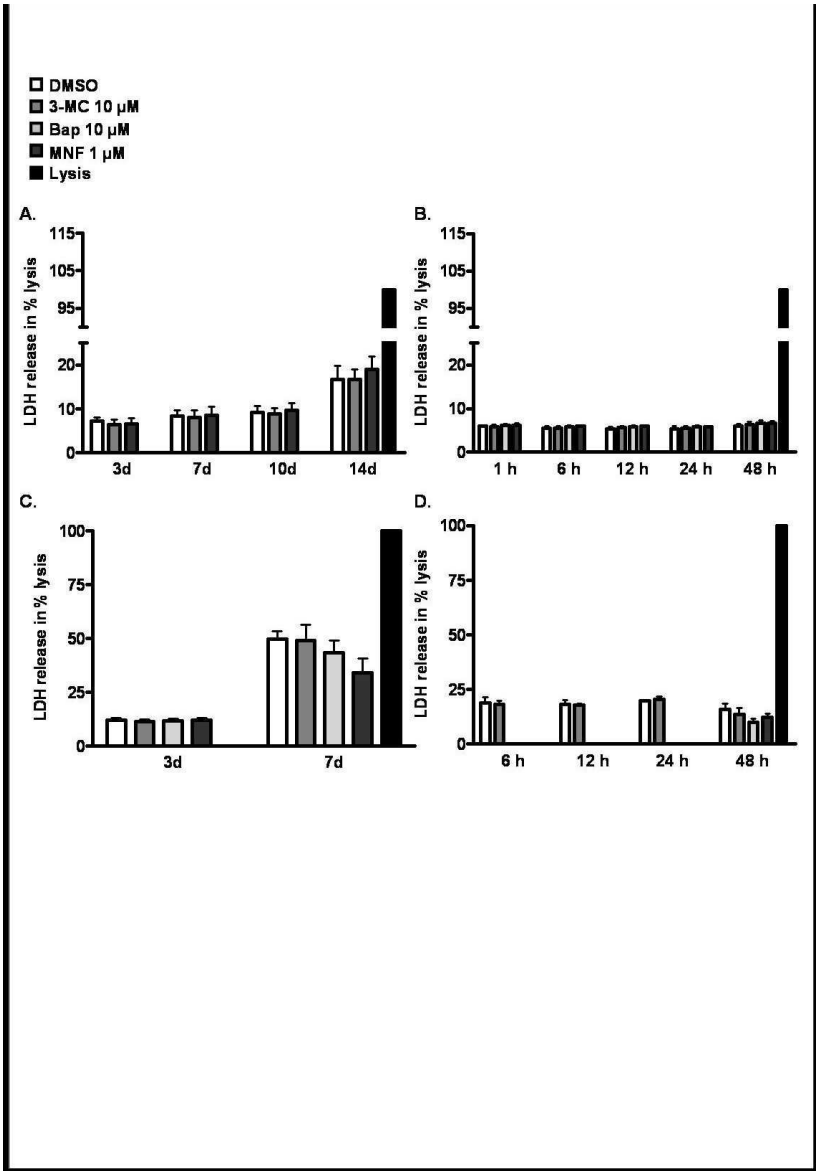


C.

	human		mouse		factor mouse/human	
	proliferating	differentiating	proliferating	differentiating	proliferating	differentiating
AhR	2.45	0.62	20.08	63.21	8.19	102.12
Arnt	12.95	13.55	716.26	1045.07	55.31	77.13
AhRR	0.94	0.61	79.21	387.55	84.24	638.85
CYP1A1	<0.001	<0.001	18.63	31.64	n.d.	n.d.
CYP1B1	0.01	0.06	146.24	834.91	16407.23	14990.75
C-Myc	5559.59	3017.13	14835.55	15967.72	2.67	5.29

Figure 3
95x141mm (300 x 300 DPI)

Supplementary Figure 1: Cytotoxicity determination by measurement of LDH-release of human neurospheres under proliferating (A) and differentiating (B) conditions and of mouse neurospheres under proliferating (C) and differentiating (D) conditions after 3-MC, B(a)P and MNF treatment for depicted times. Complete lysis of cells with a lysis buffer for 2 h at room temperature serves as positive control. Shown are means ± SEM of at least three independent experiments.



Supplementary Figure 1
96x137mm (300 x 300 DPI)

2.4 Polybrominated Diphenyl Ethers induce developmental neurotoxicity in a human in vitro model: evidence for endocrine disruption

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Environmental Health Perspectives [eingereicht am 08.09.2009; akzeptiert am 07.12.2009]

Polybromierte Diphenylether (PBDE) sind bromhaltige polyaromatische Verbindungen, die als Flammschutzmittel in vielen Kunststoffen und Textilien eingesetzt werden. Aufgrund ihrer Persistenz und ihrer Neigung zur Bioakkumulation sind sie in der gesamten Umwelt präsent und wurden in Geweben von Tier und Mensch nachgewiesen. Besorgniserregend ist, dass in den letzten Jahren die Gewebekonzentrationen im Menschen trotz EU-weitem Verbots ansteigen und besonders Säuglinge und Kleinkinder über die Muttermilch und durch im Hausstaub enthaltende PBDE exponiert sind.

Es ist bekannt, dass PBDE im Nager entwicklungsneurotoxisch sind, was sich in Hyperaktivität und verringerter Lern- und Gedächnisleistung äußert. Für den Menschen hingegen existieren bisher keine Daten zur Wirkung von PBDE auf das sich entwickelnde Gehirn. Daher war das Ziel der vorliegenden Studie (i) die Wirkung von PBDE auf die humane Gehirnentwicklung *in vitro* zu untersuchen und (ii) die zugrunde liegenden Mechanismen aufzuklären. Zur Bearbeitung dieser Aufgabenstellung wurde ein auf humanen neuralen Progenitorzellen (NPC) beruhendes Neurosphären Zellmodell verwendet und die zwei am weitesten verbreiteten PBDE Kongenere BDE-47 und BDE-99 ausgewählt.

Beide BDE beeinträchtigen in Konzentrationen zwischen 0,1 und 10 µM die für die Gehirnentwicklung essentiellen Prozesse Migration und Differenzierung ohne die Viabilität oder Proliferation der Zellen zu stören. Als empfindlichster Endpunkt stellt sich dabei die neuronale Differenzierung dar, die bereits durch Belastung mit 0,1 µM BDE-99 um ca. 40 % reduziert wird.

Zur Aufklärung des Wirkungsmechanismus wurden (i) Neurosphären mit dem Thyroidhormonrezeptor (THR) -Agonist T3 oder dem THR-Antagonisten NH-3 und den entsprechenden PBDE in Kombination belastet und (ii) Messungen der Calcium-Aktivität in den NPC nach PBDE-Exposition durchgeführt. Die Analyse der resultierenden Daten ergab, dass eine Aktivierung des THR mit T3 die Effekte der PBDE auf Migration und Differenzierung aufhebt, während der THR-anatgonist NH-3 keinen additiven Effekt ausübt. Die Belastung mit PBDE hat jedoch keinen Einfluss auf den Anstieg der Calcium-Konzentration nach Aktivierung der Calcium-Kanäle mit ATP und Acetylcholin. Aus diesen

Ergebnissen kann gefolgert werden, dass PBDE in humanrelevanten Konzentrationen die neurale Entwicklung *in vitro* stören, indem sie als endokrine Disruptoren des Thyroidhormonsignalweges wirken.

Die Verfasserin der Dissertation führte in Kooperation mit der AG Rose die Calciummessungen in den NPC durch und analysierte die Daten selbstständig. Die erhaltenen Daten führten zum Ausschluss der Hypothese, dass langfristige PBDE-Expositionen Veränderungen in Komponenten des Calciumsignalweges in NPC zur Folge haben, die Grundlage für die beobachteten Störungen in Differenzierung und Migration hätten sein können.

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Polybrominated Diphenyl Ethers Induce Developmental Neurotoxicity in a Human *in Vitro* Model: Evidence for Endocrine Disruption

Journal:	*Environmental Health Perspectives*
Manuscript ID:	09-01435-ART.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	
Complete List of Authors:	Schreiber, Timm; Institut für umweltmedizinische Forschung, Toxicology Gassmann, Kathrin; Institut für umweltmedizinische Forschung, Toxicology Götz, Christine; Institut für umweltmedizinische Forschung, Toxicology Hübenthal, Ulrike; Institut für umweltmedizinische Forschung, Toxicology Moors, Michaela; Institut für umweltmedizinische Forschung, Toxicology Krause, Guido; Heinrich-Heine-University, Department of Neurobiology Merk, Hans; RWTH Aachen, Universitaetshautklinik Crofton, Kevin; U.S. Environmental Protection Agency, Neurotoxicology Division Nguyen, Ngoc-Ha; University of California-San Francisco, Departments of Pharmaceutical Chemistry and Cellular and Molecular Pharmacology Scanlan, Thomas; Oregon Health & Science University, Department of Physiology & Pharmacology Abel, Josef; Institut für umweltmedizinische Forschung, Toxicology Rose, Christine; Heinrich-Heine-University, Department of Neurobiology Fritsche, Ellen; Institut für umweltmedizinische Forschung, Toxicology; RWTH Aachen, Universitaetshautklinik
Keywords:	Polybrominated diphenyl ethers (PBDEs) < Environmental agents, neurosphere, Development < Organ systems/disease processes, toxicology, in vitro, Human < Species, neural, Brain < Cancer/tumors < Organ systems/disease processes, neural progenitor cells


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Polybrominated Diphenyl Ethers Induce Developmental Neurotoxicity in a Human i*n Vitro* Model: Evidence for Endocrine Disruption

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Running title:

PBDEs Disrupt Human Neural Development *in Vitro*

Article Descriptor

Neurodevelopment

Keywords

Brain, development, human, in vitro, neural, neural progenitor cells, neurosphere, PBDE, toxicology

Acknowledgment and Grant information:

We acknowledge Prof. Dr. Uwe Strähle from the Karlsruhe Institute of Technology for providing the ultra-pure PBDEs used in this study. This project was funded by the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU) and the German Research Foundation (DFG) GRK1427.

Abbreviations

BDNF: Brain-derived neurotrophic factor

CaMKII: Ca^{2+}/calmodulin dependent protein kinase

CGN: Cerebellar granule neurons

EGF: Epidermal growth factor

GSH: Glutathione PDL: Poly-D lysin

hNPC: human neural progenitor cell

Nsph: Neurosphere

PBDE: Polybrominated dephenyl ether

PCB: Polychlorinated biphenyls

rhFGF: recombinant human fibroblast growth factor

T_3: Triiodothyronine

54 TH: Thyroid hormone

55 THR: Thyroid hormone receptor

Outline of second headers:

Abstract

Background: Polybrominated Diphenyl Ethers (PBDEs) are persistent and bioaccumulative flame retardants, which are found in rising concentrations in human tissues. They are of concern for human health because animal studies have shown that they possess the potential to be developmentally neurotoxic.

Objective: Because there is little knowledge on the effects of PBDEs on human brain cells, we investigated their toxic potential for human neural development *in vitro*. Moreover, we studied the involvement of thyroid hormone disruption in the effects caused by PBDEs.

Methods: We used the two PBDE congeners BDE-47 and BDE-99 (0.1 - 10 µM) which are most prominent in human tissues. As a model of neural development we employed primary fetal human neural progenitor cells (hNPCs), which are cultured as neurospheres and mimic basic processes of brain development *in vitro*: proliferation, migration and differentiation.

Results: PBDEs do not disturb hNPC proliferation, but decrease migration distance of hNPCs. Moreover, they cause a reduction of differentiation into neurons and oligodendrocytes. Simultaneous exposure with the thyroid hormone receptor (THR) agonist T_3 rescues these effects on migration and differentiation, while the THR antagonist NH-3 does not exert an additive effect.

Conclusion: PBDEs disturb development of hNPCs *in vitro* via endocrine disruption of cellular thyroid hormone signaling at concentrations which might be of relevance for human exposure.

Introduction

Polybrominated diphenyl ethers (PBDEs) are persistent and bioaccumulative flame retardants which are of concern because they are ubiquitous, potentially toxic and have been found at rapidly rising levels in humans during the past few decades (reviewed by Costa et al. 2008). PBDEs are widely used by industry as flame retardants in e.g. textiles, electrics, plastics and furniture. Over time, PBDEs diffuse out of the matrix and bioaccumulate in the environment (Schecter et al. 2006). Furthermore, these chemicals are primarily indoor pollutants and are found at high levels in household dust and other home and workplace environmental samples (Schecter et al. 2005a; Stapleton et al. 2005). Especially the abundance in household dust causes high exposure of toddlers and children (Fischer et al. 2006). PBDEs also accumulate in the human body, very high levels have recently been found in milk (Kalantzi et al. 2004; Schecter et al. 2003; 2005b), blood including fetal blood (Mazdai et al. 2003; Morland et al. 2005; Schecter et al. 2005b; Sjodin et al. 2004), placenta (Doucet et al. 2009) and adipose tissue (Johnson-Restrepo et al. 2005; She et al. 2002). Whereas levels of dioxins, dibenzofurans and PCBs have been declining in human tissues, PBDE levels have increased substantially during the past two decades (Schecter et al. 2005b; Sjodin et al. 2004).

The high levels of PBDEs in the human population, especially in infants and toddlers, are of vast concern since these compounds are chemically similar to PCBs and have been shown to be developmentally neurotoxic in rodents. Various PBDE congeners cause behavioral alterations like hyperactivity and disrupt performance in learning and memory tests in perinatally exposed mice and rats (reviewed by Costa et al. 2008).

Despite extensive information on human exposure and body burden, there is no information on possible neurodevelopmental adverse effects in humans from PBDE exposure. Therefore, any potential risk for adverse nervous system effects in humans has to be extrapolated from animal

studies (Costa et al. 2008). To facilitate PBDE hazard assessment for humans, we investigated the impact of PBDEs on human neurodevelopment *in vitro* and studied the mechanisms underlying these changes. For these analyses, we used two of the most prominent congeners found in human tissues, the tetra-brominated BDE-47 and the penta-brominated BDE-99 (reviewed by Costa et al. 2008). We investigated the effects of these PBDEs on the developmental neurotoxicity (DNT)-specific endpoints proliferation, migration and differentiation as well as on cell viability in a human model which mimics brain development *in vitro* (Fritsche et al. 2005; Moors et al. 2007; 2009; in press). Furthermore, we performed competition studies with the thyroid hormone receptor (THR) agonist triiodothyronine (T_3) and antagonist (NH-3) to investigate the involvement of thyroid hormone (TH) disruption in the observed effects by PBDEs.

Material and methods

Chemicals. BDE-47 and -99 were a kind gift from Prof. Dr. Strähle from the Karlsruhe Institute of Technology and were diluted in DMSO at stock concentrations of 1, 10 and 100 mM (Purity of both PBDEs were 98.88%). ^{14}C-BDE-47 (55 µCi/mg, stated purity > 96%) was a kind gift from Dr. Kevin Crofton from the U.S. EPA and was diluted in toluol at a stock concentration of 131 mM. T_3 (Sigma-Aldrich, Munich, Germany) and NH-3 (Nguyen et al., 2002) were diluted in ethanol (300 mM) and DMSO (1 mM), respectively.

Cell culture. Normal Human Neural Progenitor cells (hNPCs, Lonza Verviers SPRL, Belgium) generated from GW16 were cultured as free-floating neurospheres in proliferation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1)) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/ml EGF (Biosource, Karlsruhe, Germany) and 20 ng/ml rhFGF (R&D Systems, Wiesbaden-Nordenstadt, Germany), Penicillin and Streptomycin (1:100 v/v, Invitrogen GmBH, Karlsruhe, Germany) at 37°C with 7.5% CO_2 as previously described (Moors et al. 2007, 2009). Differentiation was initiated by growth factor withdrawal in

differentiation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1)) supplemented with N2 (Invitrogen) and plating onto a poly-D-lysine/laminin matrix.

Chemical exposure. For viability, migration and differentiation analyses, neurospheres were pre-incubated for 1 week with PBDEs (0.1, 1 or 10 µM) in proliferation medium, afterwards differentiation was initiated and spheres differentiated with the same concentrations of PBDEs in differentiation medium for 48 hours (migration measurements) or 7 days (differentiation analyses). This treatment scheme is supposed to imitate exposure of fetal cells during expansion and differentiation *in vivo*. For proliferation analyses, neurospheres were treated for 2 weeks with PBDEs (0.1, 1 or 10 µM) in proliferation medium. For co-treatment with T_3 or NH-3, spheres were incubated for 48 hours (migration) or 7 days (differentiation) with the indicated concentrations after differentiation was initiated.

Viability assay. Cell viability was measured using the Alamar Blue Assay (CellTiter-Blue Assay, Promega, Madison, WI, USA) which measures mitochondrial reductase activity, and the CytoTox-One Assay (Promega, Madison, WI, USA), which determines lactate dehydrogenase release, according to the manufacturer's description. In addition, to determine if PBDEs cause cell death in differentiating cells in the migration area, cells were incubated for 5 min with 0.1% trypan blue (Sigma-Aldrich, Munich, Germany). Afterwards, dead cells were counted under a light field microscope (Olympus, Hamburg, Germany).

Proliferation analyses. For proliferation analyses, spheres were cultured in proliferation medium with or without 20 ng/ml EGF/rhFGF as previously described (Moors et al. 2009). After 0 and 14 days, sphere size was determined by software analyses with the Metamorph analysis software package (Universal Imaging Corp., West Chester, PA, USA).

Migration assay. For analyses of hNPC migration, the distance from the edge of the sphere to the furthest migrated cells was measured 48 hours after initiation of differentiation at four defined positions per sphere (Moors et al. 2007).

Immunocytochemistry. After differentiating for 7 days, the cells were fixed in 2% paraformaldehyde for 30 min and stored in PBS at 4 ˚C until immunostaining was performed. For antibodies against intracellular epitopes, the fixed slides were washed two times for 5 min each in PBS containing 0.1% Triton X-100 (PBS-T). After that, slides were incubated with primary antibodies for 30 min at 37 ˚C in PBS-T containing 10% goat serum. After three additional washes with PBS, the cells were incubated for 30 min with appropriate fluorochrome labeled secondary antibodies in PBS containing 0.1 µg/ml Hoechst 33258 to label cell nuclei, followed by three washes with PBS for 10 min. After brief drying, slides were mounted with Vectashield Mounting Medium (Linaris, Wertheim, Germany). For antibodies against cell-surface epitopes the same protocol was used with the exception that PBS-T was replaced by PBS in all steps. The primary antibodies were mouse monoclonal IgG anti-β(III)-Tubulin (1:100, Sigma-Aldrich) and mouse monoclonal IgM anti-O4 (1:50, Millipore, Billerica, MA, USA). The appropriate secondary antibodies were Alexa Fluor 488 (1:250, MoBiTec, Göttingen, Germany) or Rhodamine Red coupled (1:100, Jackson ImmunoResearch, Dianova GmbH, Hamburg, Germany). For analyses, slides were examined with a fluorescent microscope (Olympus, Hamburg, Germany), and pictures were taken at the edge of the sphere with a ColorViewXS digital camera (Olympus). Stained cells were counted manually and were set into relation to the total number of nuclei in the field.

RNA preparation and reverse transcriptase-polymerase chain reaction. Total RNA was prepared from 5 differentiated neurospheres either untreated or treated with 10 µM PBDE using the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA). Real-time reverse-

transcriptase-polymerase chain reaction (RT-PCR) was performed as previously described (Fritsche et al. 2007). Primer sequences for nestin are CAGCTGGCGCACCTCAAGATG (forward) and AGGGAAGTTGGGCTCAGGACT (reverse). Primer sequences for β-actin are CCCCAGGCACCAGGGCGTGAT (foreward) and GGTCATCTTCTCGCGGTTGGCCTTGGG GT (reverse).

Calcium imaging. We treated neurospheres for 1 week with 10 µM BDE-47 or -99. Subsequently, differentiation was induced under ongoing PBDE exposure. After 24 hrs, ratiometric calcium imaging was performed using a wide-field epifluorescence system (TILL photonics, Martinsried, Germany) attached to an upright microscope (Axioskop; Zeiss, Oberkochen, Germany; 40x water immersion objective, N.A. 0.8, Olympus Europe, Hamburg, Germany). Excitation was generated by a monochromator, emission was detected by a CCD-camera (TILL imago super-VGA). Cells were passively loaded by addition of the calcium indicator dye fura-2-AM (15 µM; Teflabs; Invitrogen, Karlsruhe, Germany) for 90 minutes. Fura-2 fluorescence was alternately excited at the isosbestic point (357 nm) and at the calcium-sensitive wavelength (380 nm) and the ratio of fluorescence emission (F_{357}/F_{380}) in regions of interest positioned around cell somata was calculated. ATP and Acetylcholine (ACh) were puff-applied using a Picospritzer II (General Valve/Parker Hanifin, Flein/Heilbronn, Germany) coupled to standard micropipettes with a tip diameter of around 1.5 µm (Hilgenberg, Waldkappel, Germany) placed at a distance of approximately 10–20 µm above the cell layer or by bath application solved in ringer solution. Data analysis was performed using TILLVision and IgorPro Software (Wavemetrics, Lake Oswego, OR, USA). To determine effects on $[Ca^{2+}]_i$, we used the normalized F_{357}/F_{380} ratio for puff application. Any change in the normalized ratios to $\gtrsim$1.2 was considered as an increase and was used for further data analysis.

^{14}C-BDE-47 accumulation. After mitogen withdrawal neurospheres were allowed to attach to culture dish for 4h, afterwards cells were exposed to 1 µM ^{14}C-BDE-47. The cells were incubated for 7 days at 37°C and half of the media was changed every 2 days. At the end of the incubation period, the media was removed and cells were washed once with 500 µl PBS. Cells were lysed in 100 µl lysis buffer (Stratagene, La Jolla, CA, USA). ^{14}C-BDE-47 concentrations were determined by liquid scintillation counting in residual medium and cell lysates in Roti-Szint (Carl Roth, Karlsruhe, Germany). Intracellular ^{14}C-BDE-47 concentrations were calculated after background subtraction (same treatment without spheres) by a standard concentration curve and normalized to sphere volumes. Percent non-specific binding to the culture dish was determined by subtracting intracellular and media ^{14}C-BDE-47 from total ^{14}C-BDE-47 added to the cultures.

Statistics. For multifactor analyses ANOVA in combination with the Bonferroni post hoc test was used. Student's *t*-test was used for two group comparison. The significant value was set at $p \leq 0.05$.

Results

Effects of PBDEs on hNPC viability. To determine viability of cells, spheres were pre-incubated for 7 days with different concentrations of BDE-47 or BDE-99 under proliferating conditions followed by further differentiation in presence of PBDEs for 7 days or for 14 days as proliferating spheres. Neither mitochondrial activity nor release of lactate dehydrogenase changed significantly compared to the DMSO controls. Additionally, visual inspection of migration areas after staining with 5% trypan blue indicated that in all samples the number of dead or damaged cells lies below 1% (data not shown). Thus, BDE-47 or BDE-99 did not cause cytotoxicity of hNPC (See Supplemental Material, Figure 1).

Effects of PBDEs on hNPC proliferation. For assessment of hNPC proliferation, neurospheres were cultured with and without PBDEs (0.1 – 1 µM) for two weeks. Increase in cell number was

241 determined by measure in sphere diameter (Moors et al. 2009). In contrast to the negative control
242 without mitogens, PBDEs do not impair sphere growth over time (See Supplemental Material,
243 Figure 2).
244 *Effects of PBDEs on hNPC migration.* To measure migration, the distance between the sphere
245 edge and the furthest migrated cells was determined 48 hours after plating. Whereas the solvent
246 controls covered 942.1 ± 42.9 µm within 48 hours, BDE-47 treated hNPCs wandered 838.9 ±
247 11.6 µm (89.0 ± 1.2% of control; 0.1 µM), 755.3 ± 20.7 µm (80.2 ± 2.2% of control; 1 µM) and
248 660.7 ± 21.4 µm (70.1 ± 2.3% of control; 10 µM) and BDE-99 exposed cells migrated 781.9 ±
249 14.8µm (83.0 ± 1.6% of control; 0.1 µM), 674.5 ± 24.4 µm (71.6 ± 2.6% of control; 1 µM) and
250 609.6 ± 33.6 µm (64.7 ± 3.6 % of control; 10 µM). These data show that PBDEs reduce hNPC
251 migration in a concentration-dependant manner (Figure 1).
252 *Effects of PBDEs on hNPC differentiation.* To investigate the influence of PBDEs on
253 differentiation of hNPCs, we pre-incubated spheres with PBDEs for 7 days under proliferating
254 conditions. After 7 additional days of differentiation under PBDE exposure, we performed
255 immunocytochemical stainings for β(III)tubulin (neurons) and O4 (oligodendrocytes, Figure 2).
256 Nuclei were visualized with Hoechst showing that the number of nuclei in the differentiation
257 zones did not differ between controls and PBDE exposures (See Supplemental Material, Figure
258 3). Manual counting of immunopositive cells in blinded conditions revealed that 26.6 ± 3.2% of
259 the control cells were positive for β(III)tubulin, whereas PBDE exposure reduced these neuronal
260 cells to 21.6 ± 1.8% (0.1 µM), 16.8 ± 2.1% (1 µM) and 13.3 ± 3.0% (10 µM) for BDE-47 and to
261 16.3 ± 1.5% (0.1 µM), 13.4x ± 0.5% (1 µM) and 8.3 ± 2.0% (10 µM) for BDE-99. The effects of
262 PBDE exposure on oligodendrogenesis were stronger than on neurogenesis. While 5.7 ± 0.8% of
263 all differentiated cells were immunoreactive for O4 after 7 days of differentiation in the control
264 cultures, in the BDE-47 exposed groups only 4.5 ± 0.5% (0.1 µM), 3.9 ± 0.6% (1 µM) and 2.8 ±

0.6% (10 µM) and in the BDE-99 treated cells 4.5 ± 0.6% (0.1 µM), 2.7 ± 0.6% (1 µM) and 0.4 ± 0.4% (10 µM) stained O4 positive. Thus, PBDEs inhibit neural differentiation of hNPCs in a concentration-dependent manner.

To distinguish if PBDEs just lead to cell type specific inhibition of migration causing cells to remain within the sphere or if PBDEs lead to an actual delay in differentiation, we performed real-time RT-PCR analyses of the entire spheres for the marker of undifferentiated progenitor cells nestin. Control spheres display only a weak expression of nestin after 7 days of differentiation. In contrast, BDE-47 and BDE-99 increase in nestin expression 4- and 5-fold showing that PBDEs delay differentiation of hNPCs (Figure 3B).

PBDEs interfere with THR signal transduction. To study involvement of endocrine disruption in the observed PBDE effects, we employed the THR agonist T_3 and the antagonist NH-3. After initiating differentiation, we exposed the spheres to 10 µM BDE-47 or BDE-99 with or without 3 nM T_3 or 1 µM NH-3. Stimulation with T_3 alone increases migration distance significantly to 1181.6 ± 7.4 µm (121.1 ± 0.6% of control) in comparison to 975.6 ± 7.4 µm in the controls (Figure 3A). In contrast, NH-3 inhibits migration of hNPCs to 735.2 ± 7.2 µm, (78.2 ± 1.8% of controls). Co-administration of BDE-47 or BDE-99 and T_3 rescued the inhibitory effects of PBDEs completely, the cells migrate over a distance of 1107.4 ± 8.4 µm (113.5 ± 1.2% of control; BDE-47) and 1109.2 ± 18.6 µm (113.8 ± 2.6% of control; BDE-99). In contrast, co-treatment of PBDEs with NH-3 does not add an effect indicating that these substances inhibit migration through an identical mechanism.

For addressing the question if endocrine disruption of the TH system is also responsible for PBDE-induced changes in differentiation, we analyzed nestin expression 7 days after co-treatment with PBDEs and T_3 or NH-3. T_3 rescued the PBDE-induced cellular increase in nestin expression, while NH-3 show no additive effects in combination with PBDEs (Figure 3B). These

289 results support the notion that PBDEs delay neural differentiation by interfering with cellular
290 THR signaling.
291 *PBDEs do not influence calcium signaling.* We treated neurospheres for 1 week with 10 µM
292 BDE-47 or -99. Subsequently, differentiation was induced under ongoing PBDE exposure. After
293 24 hrs, neurospheres were stimulated with 1 mM ATP or 500 µM ACh to induce calcium
294 signaling. ATP causes a $[Ca^{2+}]_i$ increase in 94.7 ± 4.1% of all cells with a maximal amplitude of
295 1.5 ± 0.1, whereas ACh causes a $[Ca^{2+}]_i$ elevation in 29.2 ± 3.6% of all cells with an amplitude of
296 1.32 ± 0.0. However, BDE-47 and BDE-99 do not change number of responding cells or
297 amplitude of response (Figure 4).
298 *PBDEs accumulate in hNPCs.* Exposure of hNPCs to 1 µM ^{14}C-BDE-47 for 7 days resulted in a
299 cellular concentration of 61.16 ± 6.34 µM, which equals an accumulation factor of 60. Only 2%
300 of ^{14}C-BDE-47 remained in the media, meaning that 91% of ^{14}C-BDE-47 was bound to the
301 culture dish (See Supplemental Material, Figure 4).
302 **Discussion**
303 Human exposure to brominated flame retardants is of concern because PBDEs impair
304 neurodevelopment in animals, rising concentrations of these compounds are found in human
305 tissues, and nothing is known about their developmentally neurotoxic effects in men (Costa et al.
306 2008). Moreover, the mechanisms by which PBDEs interfere with brain development are
307 inscrutable. For shedding light onto the consequences that PBDE exposure cause to human
308 developing brain cells, we studied their effects on the development of hNPCs *in vitro*. These cells
309 are primary human fetal neuroprogenitors and grow as three-dimensional, complex cellular
310 systems, called neurospheres, in culture. Recently, we have established several endpoints for
311 DNT testing in such neurospheres: proliferation, migration, differentiation and apoptosis by
312 employing endpoint-specific controls (Moors et al. 2007; 2009). We showed that this neurosphere

313 system is able to reveal exogenously-induced disturbances in these basic processes of brain
314 development. Therefore, here we apply this novel testing method to unravel the DNT potential of
315 PBDEs for such developing human cells.
316 PBDEs (0.1 – 10 µM) are not cytotoxic for proliferating or differentiating hNPCs over a period of
317 2 weeks as we demonstrated by different independent methods (See Supplemental Material,
318 Figures 1 & 2). In contrast to our findings, the technical PBDE mixture DE-71 (>20 µM) caused
319 cell death in rat cerebellar granule cells (Reistad et al. 2006), while BDE-99 (>25 µM) induced
320 cytotoxicity in human astrocytoma cells (Madia et al. 2004). BDE-47 was reported to cause cell
321 death in hippocampal neurons (41.2 µM), human neuroblastoma cells (>5 µM), and human fetal
322 liver hematopoietic cells (>50 µM) (He et al. 2008, He et al. 2008; Shao et al. 2008). Moreover,
323 the higher brominated BDE-209 (>10 µM) was toxic to human hepatoma cells HepG2 (Hu et al.
324 2007). Cause for cytotoxicity in all these different cell models was induction of apoptosis.
325 Giordano et al. (2008) demonstrated that DE-71 exposure induces oxidative stress. That this
326 production of reactive oxygen species is responsible for DE-71-induced apoptosis of rat
327 cerebellar granule neurons (CGN) became obvious by showing that intracellular glutathione
328 (GSH) content is a most important determinant of CGN susceptibility to DE-71 neurotoxicity.
329 Moreover, transgenic rat neuron – astrocyte co-cultures with proficient vs. deficient GSH
330 synthesis supported these findings as astrocytes rich in GSH protected neurons against DE-71-
331 induced neurotoxicity while astrocytes with poor GSH content did not (Giordano et al. 2009).
332 Protection of neurons against PBDE-induced cytotoxicity by presence of astrocytes is thus the
333 probable reason for differentiated hNPC insensitivity towards PBDE-dependent cell death while
334 proliferating precursors are known to posses better defense mechanisms than postmitotic neural
335 cells (Madhavan et al. 2006). Moreover, such human co-culture systems like differentiated
336 neurospheres, where the three major cell types of the brain are present in ‘physiological’ ratios,

337 seems to be a superior *in vitro* method for assessing hazards of chemicals to humans when
338 compared to simple monolayer cell lines.
339 Neurosphere proliferation assessed by monitoring of sphere diameter (Moors et al. 2009) was
340 also not affected by presence of BDE-47 or -99 (See Supplemental Material, Figure 2). This is in
341 agreement with results from a T-screen assay (Gutleb et al. 2005), a functional assay based on T_3-
342 dependent cell proliferation of the rat pituitary tumor cell line GH3, where also no effects of these
343 two congeners on proliferation were observed (Hamers et al. 2006). In contrast, one of the
344 hydroxylated BDE-47 metabolites, 2-OH-BDE-47 (5-10 µM) inhibited proliferation of the
345 H295R adrenocortical carcinoma cell line (Song et al. 2009). However, it is to consider that this
346 concentration of reactive metabolite causing an antiproliferative effect in these cells is high and
347 the authors did not investigate effects of the parent compound. Inhibition of proliferation by
348 BDE-47 was also seen in 5L rat hepatoma cells. However, the authors demonstrated that
349 arylhydrocarbon receptor (AhR) activation by the BDE-47 contaminant 1,2,3,7,8-pentabromo-
350 dibenzofuran was rather responsible for the effects on proliferation than BDE-47 itself (Wahl et
351 al. 2008). Highly purified BDE-47 does not stimulate the AhR (Peters et al. 2004). This supports
352 our data because PBDEs used in this study are contaminant-free. Increased proliferation was
353 observed in DE-71-treated MCF-7 breast cancer cells. This stimulation was estrogen receptor-
354 dependent and thus DE-71 acts as an endocrine disruptor in this estrogen receptor-positive cell
355 line (Mercado-Feliciano and Bigsby, 2008). Hence, interaction of PBDEs with cell proliferation
356 seems to be congener and cell type specific. So far, all data available on this topic have been
357 obtained in tumor cells. This is - to our knowledge - the first report employing normal human
358 cells or human stem/progenitor cells for determining effects of PBDEs on cell proliferation.
359 In contrast to proliferation, both investigated PBDE congeners inhibit migration and
360 differentiation of hNPCs significantly in a concentration-dependent manner (Figure 1 & 2).

Consequences of PBDE exposure for cell migration have not been investigated so far in any cell type *in vitro* or *in vivo* and thus this is the first report showing that these chemicals have the ability to interfere with human progenitor cell motility. However, a recent proteomics study by Alm et al. (2008) suggests that PBDE exposure might cause disturbances in cell motility also *in vivo*. In this work, a single dose of BDE-99 given on mouse PND10 caused changes in brain protein expression after 24 hrs and one third of those proteins were related to the cytoskeleton including actin. The importance of the actin cytoskeleton for neuronal migration has been reviewed extensively (Luo 2002) leaving room for the speculation that PBDEs might interfere with migration through alteration of cytoskeleton-related protein expression. Similar to PBDE effects on neural migration, to our knowledge, consequences of PBDE exposure for neural differentiation have not been studied so far. Therefore, this is the first report showing that this group of flame retardants can directly interfere with birth of neurons and oligodendrocytes in this human *in vitro* model. Interference of BDE-47 with neuronal differentiation was also suggested in the fathead minnow because this congener reduced basic transcription element-binding protein (BTEB) expression in their brains (Lema et al. 2008). BTEB is known to be involved in neural differentiation in rodents (Denver et al. 1999). Moreover, BDE-99 altered protein expression of GAP-43 and brain-derived neurotrophic factor (BDNF) in developing rodent brains (Alm et a. 2008). Both proteins are closely related to neural development and plasticity (reviewed by Strittmatter et al. 1992 and Binder and Scharfman 2004). If PBDEs disturb brain development by interfering with migration and differentiation not only *in vitro* but also *in vivo*, needs to be investigated by appropriate animal experiments. However, as our study was performed in normal cells of human origin possible species differences should first be addressed by migration and differentiation analyses in rodent neurospheres. Moreover, the developmental stage of cells needs

to be considered for rodent *in vitro* and *in vivo* analyses: hNPC generated from GW16 correspond approximately to E16 to PND3 of mouse development.

During normal development, neural migration and maturation of neural and glial cells are guided by TH (Alvarez-Dolado et al. 1999; Wong and Leung 2001). Therefore, hypothyroidism during development causes a large number of neuroanatomical and behavioral effects (Haddow et al. 1999; Schalock et al. 1977; Zoeller and Crofton 2005). Due to similar neurobehavioral alterations observed after PBDE exposure in rodents, endocrine disruption of the TH system by PBDEs has been studied intensively. Hypothyroidism of dams and/or offspring was found in a variety of different studies after pre- or postnatal exposure (reviewed by Costa et al. 2008). This reduction in serum T_4 or T_3 levels is thought to be caused by induction of the phase II enzyme UDP-glucuronosyl transferase causing accelerated TH metabolism (Zhou et al. 2002) and by displacing TH from binding to its plasma transport protein transthyretin (Meerts et al. 2000). However, behavioral toxicity of BDE-47 without alterations in serum T_4 and T_3 levels were also observed suggesting that PBDE cause toxicity by a mechanism beyond changes in body TH homeostasis (Gee et al. 2008; Gee and Moser 2008). These studies might be explained by the data generated in our experiments because the two PBDE congeners BDE-47 and -99 directly disturb migration and delay differentiation of hNPCs *in vitro* by endocrine disruption of cellular TH signaling. We confirmed this by two observations: (i) PBDE actions are completely antagonized by co-treatment of neurospheres with T_3 and (ii) simultaneous administration of PBDE and the THR antagonist NH-3 did not cause an additive effect. Although NH-3 binds to THRα as well as THRβ, it has a higher affinity to THRβ (Nguyen et al. 2002). As inhibition of THRα leads to decreased proliferation of avian neurogenic precursors (Lezoualc´h et al. 1995; but PBDEs do not interfere with hNPC proliferation) and the induction of THRβ induces neural differentiation (Jones et al. 2003; Lebel et al. 1994; and PBDEs disturb neural differentiation of hNPCs), it is

likely that PBDEs interfere with THRβ signaling of hNPCs. However, reporter gene analyses in THRα and β overexpressing CHO cells revealed that both PBDE congeners did not act as agonists or antagonists of THRα or β. One possible reason for discrepancies seen between our study and results in this overexpression system might be that PBDEs act via disturbance of recruitment of THR co-factors. In primary cells, receptors, co-factors and responsive elements are present at a fine-tuned equilibrium which is not the case in transfected cells which overexpress only certain players of the machinery. That nuclear hormone receptor co-factors might be crucial in endocrine disruption by polyhalogenated aromatic compounds was already discussed in our previous work where we found that the non-coplanar PCB118 induced oligodendrocyte differentiation in hNPCs (Fritsche et al. 2005). This *in vitro* work represented the finding *in vivo* that Aroclor treatment led to an increased expression of TH-dependent genes such as RC3/neurogranin and myelin basic protein in fetal rat brains (Zoeller et al. 2000). To our knowledge, an equivalent rodent study has not been performed employing PBDEs yet. Only in fish, in the fathead minnow, TH disruption on the basis of THR-dependent gene expression was observed after BDE-47 exposure (Lema et al. 2008). Thus, this is the first work showing that PBDEs can directly interfere with cellular TH signaling in human neural cells.

Besides endocrine disruption we also investigated if PBDEs disturb calcium homeostasis. Calcium signaling is a key player in developmental processes (Greer and Greenberg 2008; Ciccolini et al. 2003) and BDE-47 disturbs calcium homeostasis in a rat pheochromocytoma cell line (PC12 cells) after 20 min of exposure (Dingemans et al. 2008). BDE-47 exerts similar short term effects on hNPCs (Gassmann et al., submitted). To test if long term exposure (1 week) towards PBDEs also influences calcium signaling and thus contributes to the developmentally neurotoxic effects of PBDEs in hNPC, we measured calcium signaling in PBDE treated hNPCs stimulated with ATP or ACh. Neither BDE-47 nor -99 influences the response of the cells towards

these stimuli indicating that they do not alter expression of proteins involved in Ca^{2+} flux. Recently it was shown that BDE-203 and BDE-206 increase CaMKII expression in mouse hippocampus (Viberg 2009). If PBDEs interfere with downstream targets of calcium signaling in hNPCs like CaMKII or calcineurin has to be further elucidated.

In summary, BDE-47 and -99 disturb neural migration and differentiation in a human *in vitro* model for brain development by disruption of cellular TH signaling. The question is now how the lowest observed effect levels from this study relate to actual human PBDE exposure. Therefore, we measured intracellular PBDE concentrations by employing ^{14}C-BDE-47. Because medium concentration-dependent intracellular PBDE accumulation *in vitro* follows a linear kinetic (Mundy et al. 2004) and neurosphere material is very little, we only measured 1 µM ^{14}C-BDE-47 medium concentration. After 7 days of differentiation PBDE accumulation is approximately 60-fold. This data supports other *in vitro* data where PBDEs accumulate up to 100-fold in neuronal cells (Mundy et al. 2004). It also reflects PBDE accumulation in PND10 and 19 mouse brains after 7 days *in vivo* (20 – 140-fold) that we calculated from a study of Viberg et al. (2003). PBDE exposure of human infants through breast milk is up to 4000 ng/kg/day (Jones-Otazo et al. 2005). Assuming an average molecular weight of 500 g/mol for PBDEs this equals an exposure of 8 nM. Taken into account that there is a 60-fold increase in tissue concentration of PBDEs in our human *in vitro* system and up to an 140-fold increase in brain tissue in mice after oral exposure *in vivo* (Viberg et al. 2003), infant exposure could result in a brain concentration of 0.5 – 1.1 µM. Considering that 0.1 µM BDE-99 (approx. 6 µM tissue concentration) decreases neuronal differentiation by ~ 40% (Fig. 2), current PBDE exposure levels are likely to be of concern for human health.

454 Assessing subtle changes in human IQ or behavior in epidemiological studies is not trivial and
455 needs large numbers of study subjects. Such investigations are needed to reveal if PBDEs as a
456 hazard identified in this study actually pose a risk for human brain development *in vivo*.

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626 Figure legends

627

628 Figure 1: PBDEs inhibit migration of neural progenitor cells. Phase contrast images (A) and
629 respective quantification (B) of cell migration. Migration distance was measured at four defined
630 spots from the edge of the sphere to the furthest migrated cell after 48 hours. All data are mean ±
631 SEM of three independent experiments (5 spheres/experiment). Scale bar: 200 µm. *p*-value ≤
632 0.05.

633

634 Figure 2: PBDEs inhibit differentiation of hNPCs. (A) Representative photomicrographs of
635 hNPCs after 7 days of differentiation. Cells were stained with antibodies against β(III)-Tubulin
636 for neurons and O4 for oligodendrocytes. Cell nuclei were counterstained with Hoechst. (B)
637 Quantification of immunostainings after PBDE treatment. All data are mean ± SEM of three
638 independent experiments (5 spheres/experiment). Scale bar: 50 µm. *p*-value ≤ 0.05.

639

640 Figure 3: PBDEs disrupt cellular thyroid hormone signaling. (A) hNPC migrated for 48 hours in
641 presence of the indicated substances. Migration distance was quantified. (B) hNPCs differentiated
642 for 7 days in presence of the indicated substances. Real-time PCR analyses were quantified with
643 a product-specific copy number standard and normalized for ß-actin expression. All data (percent
644 of DMSO control) are shown as mean ± SEM of three independent experiments (5
645 spheres/experiment). *p*-value ≤ 0.05. * significant compared to the control. † significant
646 compared to the respective PBDE treatment.

647

648 Figure 4: Long-term PBDE exposure does not interfere with calcium signaling. Neurospheres
649 were incubated with 10 µM BDE-47 or -99 for 7 days under proliferating conditions and for 1

additional day during differentiation. Afterwards, hNPCs were loaded with the fura-2-AM dye and puff-exposed towards 1 mM ATP or 500 µM Ach. After excitation, the ratio of fluorescence emission (F_{357}/F_{380}) in regions of interest positioned around cell somata was calculated. Any change in normalized ratios (F_{357}/F_{380}) $\geq$ 1.2 was considered as an increase and used for further analysis. All data are mean ± SEM of three independent experiments (5 spheres/experiment). *p*-value $\leq$ 0.05.

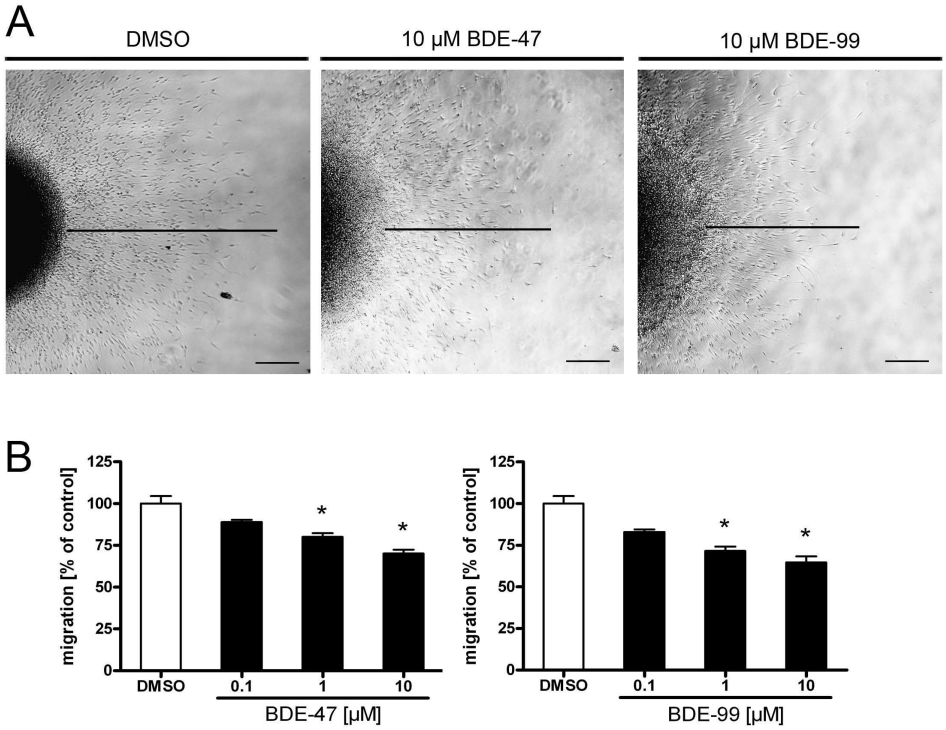

Figure 1
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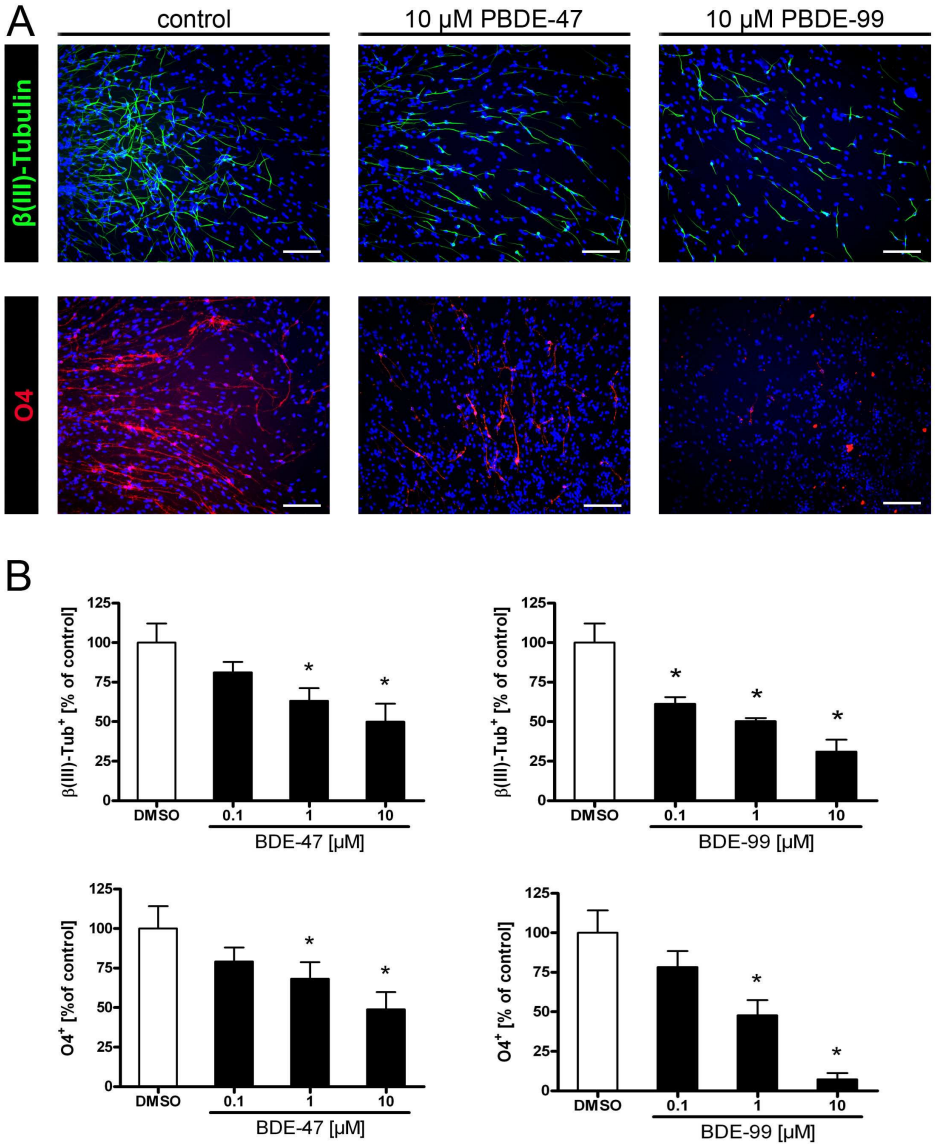

Figure 2
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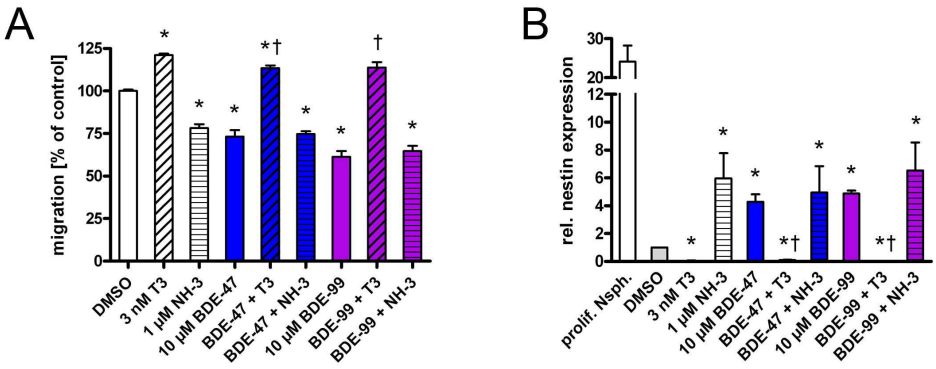

Figure 3
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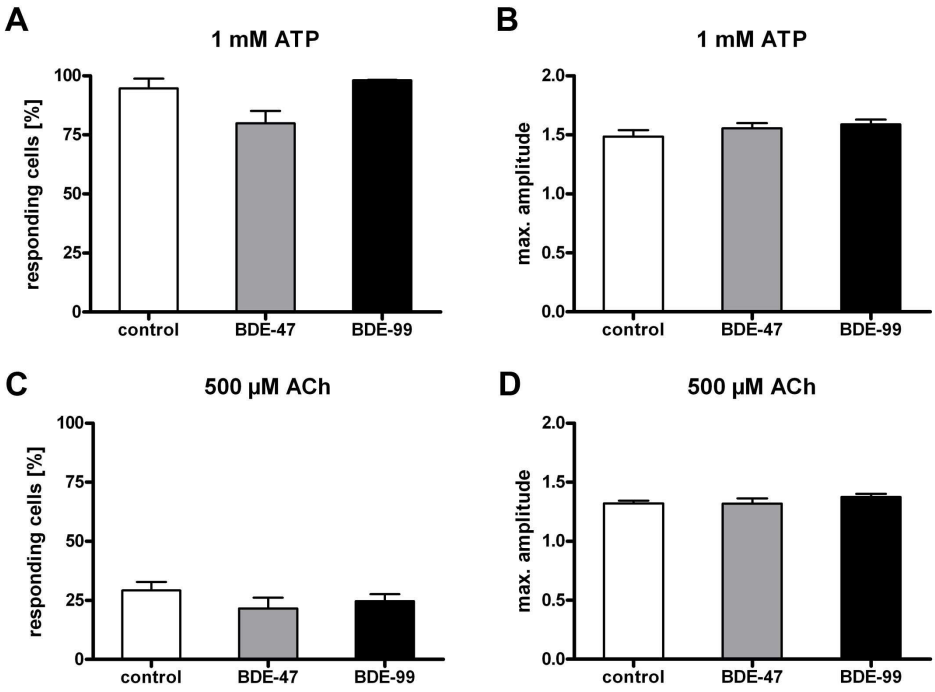


Figure 4

188x138mm (300 x 300 DPI)

Supplemental Material

Figure legends

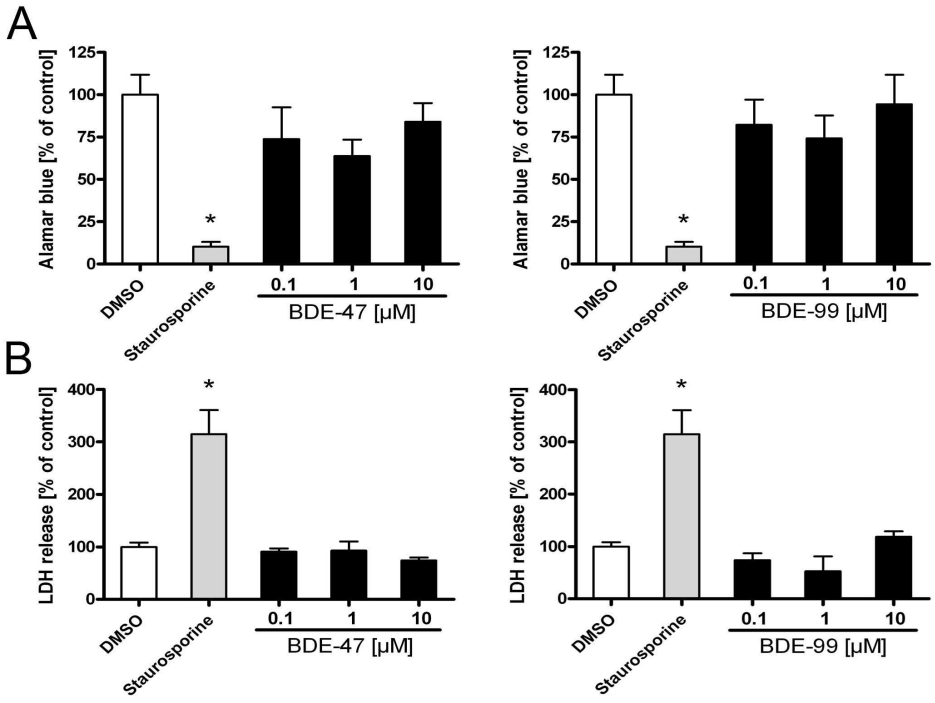
Supplementary Figure 1: PBDEs are not cytotoxic to hNPCs. After 1 week of exposure towards PBDEs in proliferation medium, spheres were plated onto a PDL/laminin matrix under mitogen withdrawal in further presence of PBDEs. Cytotoxicity of PBDEs were measured by the Alamar Blue Assay (A) or lactate dehydrogenase release (B). All data are mean ± SEM of three independent experiments (5 spheres/experiment). p-value ≤ 0.05.

Supplementary Figure 2: PBDEs have no effect on hNPC proliferation. Neurospheres were cultured in proliferation medium in presence or absence of PBDEs. Proliferation was quantified by assessment of sphere diameter over time. Growth was determined as difference between the diameters after 0 and 14 days and shown as % of control. All data are mean ± SEM of three independent experiments (6 spheres/experiment). p-value ≤ 0.05.

Supplementary Figure 3: PBDEs do not affect cell number in the migration area of hNPCs. Cell nuclei were stained with Hoechst and number of cells per image was determined. All data are mean ± SEM of six independent experiments (5 spheres/experiment). p-value ≤ 0.05.

Supplementary Figure 4: ^{14}C-BDE-47 accumulates in hNPCs. After mitogen withdrawal neurospheres were allowed to attach to culture dish for 4h, afterwards cells were exposed to 1 µM ^{14}C-BDE-47 for 7 days and half of the media was changed every 2 days. ^{14}C-BDE-47 concentrations were determined by liquid scintillation counting in residual medium and cell

lysates. Intracellular ^{14}C-BDE-47 concentrations were calculated after background subtraction (same treatment without spheres) by a standard concentration curve and normalized to sphere volumes. Percent non-specific binding to the culture dish was determined by subtracting intracellular and media ^{14}C-BDE-47 from total ^{14}C-BDE-47 added to the cultures. All data are mean ± min/max of two independent experiments.



Supplementary Figure 1
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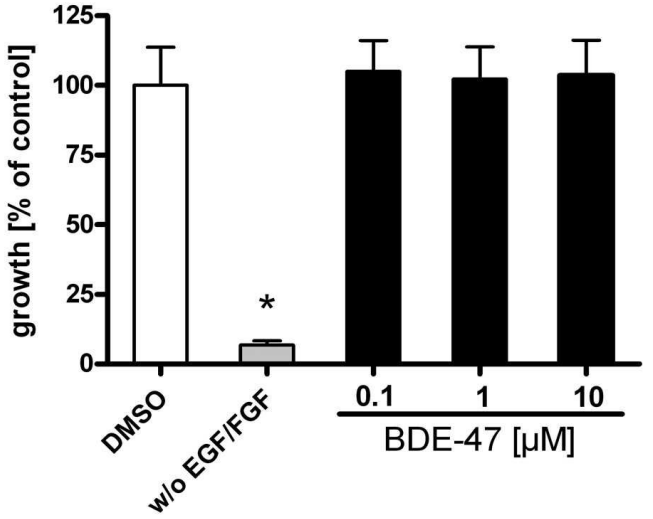

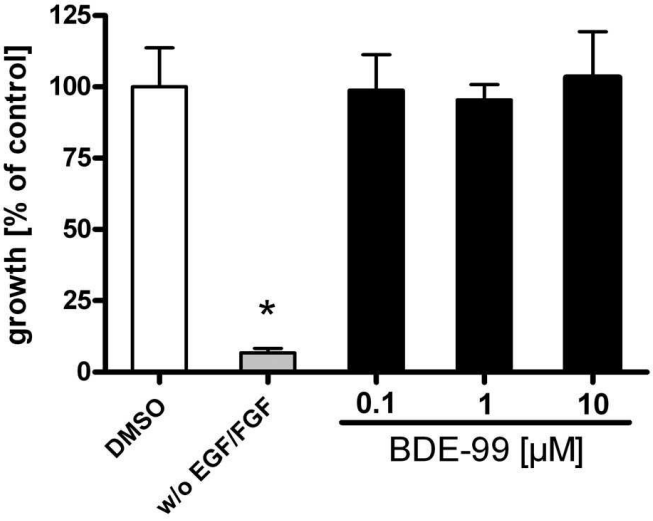

Supplementary Figure 2
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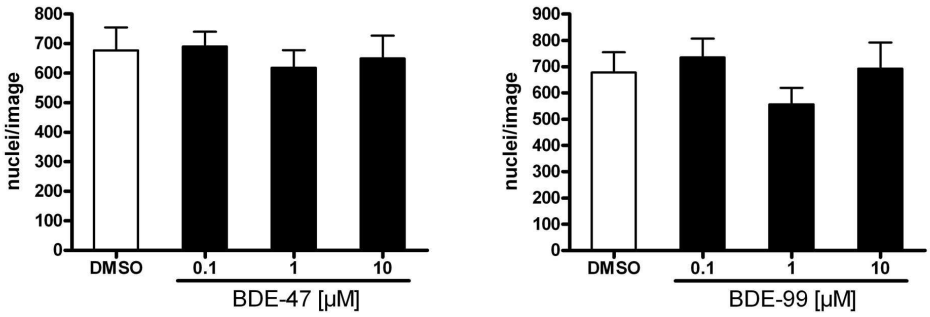

Supplementary Figure 3
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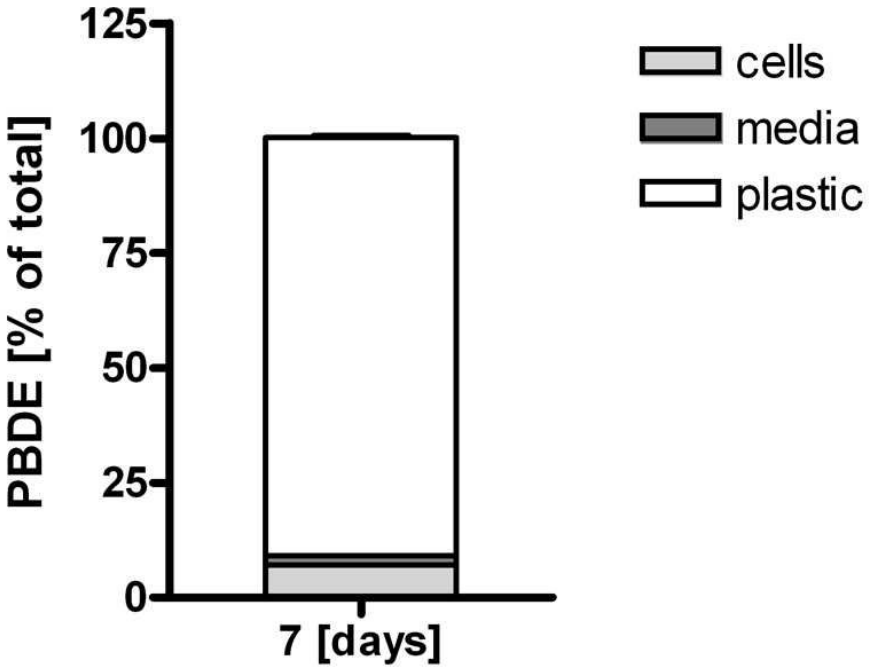

Supplementary Figure 4
71x58mm (300 x 300 DPI)

2.5 Publikation 5: BDE-47 and its hydroxylated metabolite 6-OH-BDE47 modulate calcium homeostasis in primary human neural progenitor cells

Gassmann K, Krause G, Dingemans MML, Schreiber T, Abel J, Bergman A, Merk HF, Moors M, Rose CR, Westerink RHS, Fritsche E.

[Environmental Health Perpectives in Vorbereitung]

Polybromierte Diphenylether (PBDE) sind bromhaltige polyaromatische Verbindungen, die als Flammschutzmittel in vielen Kunststoffen und Textilien eingesetzt werden. PBDE werden im Körper durch Oxidation metabolisiert. Experimentelle Studien belegen die Entstehung von hydroxylierten PBDE-Metaboliten nach Inkubation von PBDE-Kongeneren in induzierten Leber- Mikrosomen und auch *in vivo* konnte die Existenz hydroxylierter PBDE-Metabolite in Fäzes und Urin von Ratten und Mäusen nachgewiesen werden.

In der Ratten-Phäochromozytomzellinie PC-12 führt besonders der oxidierte Metabolit des BDE-47 (6-OH-BDE-47) zu einem Anstieg der Calciumkonzentration in der Zelle. Calcium reguliert wichtige physiologische und neuronale Funktionen, wobei die intrazelluläre Calciumkonzentration durch diverse Calcium-Kanäle und -Transporter streng kontrolliert wird. Eine Störung der Calciumhömeostase kann sich daher potentiell negativ auf eine ganze Reihe von zellbiologischen Prozessen im Gehirn wie z.B. Proliferation, Differenzierung, Apoptose, synaptische Plastizität und Neurotransmission auswirken. In der vorhergehenden Publikation wurde ausgeschlossen, dass eine Langzeit-Applikation von PBDE einen Einfluss auf die Aktivierbarkeit von Calciumkanälen hat. Ziel der vorliegenden Publikation war es daher die Effekte von kurzzeitiger PBDE Applikation auf die Calcium-Homöostase in humanen Neurosphären zu untersuchen. Zur Messung der intrazellulären Calciumkonzentration wurde der fluoreszierende Ca^{2+}-Chelator Fura-2 verwendet.

Voraussetzung für diese Untersuchungen ist ein funktionelles Calciumsystem in Neurosphären, dass durch extrazelluläre Stimuli aktivierbar ist. Sowohl die Applikation von unspezifischen Stimuli wie ATP als auch von spezifischen Neurotransmittern wie Acetylcholin und Glutamat erhöht die intrazelluläre Calciumkonzentration. Der Anteil der reagierenden Zellen ist dabei im Falle von Glutamat von der Differenzierungszeit abhängig, was vermuten lässt, dass ein gewisser Reifungsgrad der Zellen zur Reaktion auf diesen Stimulus Voraussetzung ist. Die Anzahl aktiver Zellen und die Amplituden des Calciumsignals sind in allen anderen Fällen nicht von der Differenzierungszeit beeinflusst. Weiterführende Patch-Clamp Untersuchungen konnten sowohl Einwärts- als auch Auswärtsströme identifizieren, aber in keiner der gemessenen Zellen konnte ein Aktionspotential ausgelöst werden, was auf einen geringen neuronalen Reifungsgrad des Systems rückschließen lässt.

Ausgehend von diesen Voruntersuchungen wurden daraufhin Neurosphären akut mit BDE-47 und seinem Metaboliten 6-OH-BDE-47 belastet und die entsprechenden intrazellulären Calciumkonzentrationsänderungen analysiert. Es zeigte sich, dass sowohl BDE-47 als auch 6-OH-BDE-47 eine Calciumkonzentrationserhöhung in den NPCs auslösen. Ähnlich wie in der Studie mit den PC12-Zellen ist der hydroxylierte Metabolit deutlich potenter und führt schon in einer Konzentration von 0,2 µM zu signifikanten intrazellulären Konzentrationsänderungen. Weiterführende Experimente mit verschiedenen Blockierungsreagenzien führten zu dem Ergebnis, dass der Anstieg des intrazellulären Calciumspiegels in den NPCs nach BDE-Belastung sowohl durch Einstrom aus dem umgebenen extrazellulären Medium als auch durch Entleerung intrazellulärer Speicher wie des endoplasmatischen Retikulums und der Mitochondrien erfolgt. Im Gegensatz zu PC-12 Zellen scheint es im humanen System darüber hinaus aber noch einen weiteren Speicher zu geben, der nach PBDE-Applikation Calcium in das Zytoplasma ausschüttet.

Die Verfasserin der Dissertation führte in Kooperation mit der AG Rose sämtliche Calciummessungen zur allgemeinen elektrophysiologischen Charakterisierung der Neurosphären durch und analysierte die Daten selbstständig. Die so generierten Daten waren die Grundvoraussetzung für die Untersuchung der spezifischen Effekte von PBDE auf die Calciumhomöostase der NPC.

BDE-47 and its hydroxylated metabolite 6-OH-BDE47 modulate calcium homeostasis in primary human neural progenitor cells

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Short running title

BDE-47 modulates Ca^{2+} homeostasis in hNPCs

Key words

Polybrominated diphenyl ether, calcium, human, brain, neural, brominated flame retardant, neurotoxicity, intracellular calcium stores, neurosphere

Acknowledgments/grant support

We acknowledge the expert technical help of Claudia Roderigo. Parts of this project were funded by the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety (BMU) and by the German Research Foundation (DFG GRK1427).

Abbreviations

ER: Endoplasmatic reticulum

FCCP: carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone

hNPC: Human neural progenitor cell

LDH: Lactate-dehydrogenase

LTP: Long term potentiation

PCB: Polychlorinated biphenyl

PBDE: Polybrominated diphenyl ether

SERCA: Sarcoplasmatic/endoplasmatic Ca^{2+}-ATPase

SOCE: store-operated Ca^{2+} entry

TG: Thapsigargin

Outline of manuscript section headers

Abstract

Background: Polybrominated Diphenyl Ethers (PBDEs) are bioaccumulating and persistent flame retardants, which are found in rising concentrations in human tissue. Animal studies indicate that they are developmentally neurotoxic. Oxidative metabolism of PBDEs may lead to toxification of the parent compound.

Objective: We investigated the effects of 2,2′,4,4′ tetrabromodiphenyl ether (BDE-47) and its hydroxylated metabolite 6-hydroxy-2,2′,4,4′tetrabromodiphenyl ether (6-OH-BDE-47) on intracellular Ca^{2+} $[Ca^{2+}]_i$ homeostasis and signalling in human neural progenitor cells (hNPCs).

Methods: We measured membrane potential and ion currents using whole cell patch-clamp recordings. Intracellular Ca^{2+} imaging was performed with the fluorescent Ca^{2+}-sensitive dye Fura-2.

Results: Acute exposure of hNPCs to BDE-47 (2 μM) or 6-OH-BDE-47 (0.2 μM) results in a significant increase in $[Ca^{2+}]_i$. 6-OH-BDE-47 (20 μM) causes an initial transient increase and a late persistent increase in $[Ca^{2+}]_i$ with a shift of the baseline. Emptying intracellular Ca^{2+} stores by employing the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and the SERCA inhibitor thapsigargin (TG) revealed that PBDE-dependent initial transient Ca^{2+} increase depends on intracellular Ca^{2+} release from the ER, whereas the shift of the baseline is mainly due to Ca^{2+} release from mitochondria. The late persistent increase in $[Ca^{2+}]_i$ seems to be caused by influx of extracellular Ca^{2+}.

Conclusion: Both, parent BDE-47 and the hydroxylated metabolite 6-OH-BDE-47 disturb Ca^{2+} homeostasis of hNPCs. However, the hydroxylated metabolite exerts a higher potency than the parent compound. These findings indicate that oxidative metabolism of PBDEs seems to increase their toxic potential for human neural progenitor cells.

Introduction

Polybrominated diphenyl ethers (PBDEs) are nowadays ubiquitously present in the environment, in animals and in humans. The current greatest concern for potential adverse health effects of PBDEs relates to their developmental neurotoxicity (DNT; rev. in Costa and Giordano 2007). Previous data demonstrated that high and low doses of PBDEs administered during pregnancy or postnatally during the brain growth spurt alter the neurobehaviour of the offspring in rodents ranging from altered motor activity over changes in sweet preference to impairment of cognitive functions (Cheng et al. 2009;Eriksson et al. 2002;Lilienthal et al. 2006;Suvorov et al. 2009;Viberg et al. 2003). One of the proposed mechanisms how PBDEs exert DNT is disruption of calcium (Ca^{2+}) homeostasis as shown in rat pheochromocytoma (PC12) cells, human neuroblastoma (SH-SY5Y) cells and in isolated rat brain organells *in vitro* (Dingemans et al. 2008;Kodavanti and Ward 2005;Yu et al. 2008). Ca^{2+}, an early-response second messenger, plays a key role in a number of physiological processes including cell proliferation, differentiation and apoptosis (Berridge et al. 2000;Ciccolini et al. 2003;van Breemen and Saida 1989). The complex spatial and temporal properties of intracellular Ca^{2+} signals also encode a wide variety of specific neuronal processes including dendritic spine growth, synaptic plasticity and neurotransmission. Ca^{2+} influx as well as Ca^{2+} release from intracellular Ca^{2+} stores such as endoplasmic reticulum (ER) or mitochondrial stores define the magnitude, time course, and spatial spread of the Ca^{2+} signal (Berridge 1998;Delmas and Brown 2002).

2,2´,4,4´-tetrabromodiphenyl ether (BDE-47) is the predominant PBDE congener in human blood samples (Hites 2004). The conversion of PBDEs to hydroxylated metabolites was confirmed by recent toxicokinetics studies (Huwe et al. 2007;Malmberg et al. 2005;Marsh et al. 2006;Staskal et al. 2006b) and high amounts of hydroxylated metabolites are found in blood of humans (Athanasiadou et al. 2008), including fetal blood (Qiu et al. 2009). *In vitro* studies on endocrine parameters and on calcium homeostasis in PC12 cells revealed that such hydroxylated metabolites of PBDEs are more potent then their parent compounds (Canton et al. 2006;Dingemans et al. 2008;Harju et al. 2007;Meerts et al. 2001). These findings suggest that bioactivation by oxidative metabolism might add to the neurotoxic potential of PBDEs.

With regard to hazard assessment, there is no data on the effects of PBDEs on human brain cells so far. We have developed a cell culture system which is based on normal human neural progenitor cells (hNPCs) and mimics basic processes of brain development *in vitro*: proliferation, migration, differentiation and apoptosis. We employ this three-dimensional, neurosphere-based model to detect developmental neurotoxicity of exogenous noxae (Fritsche

et al. 2005;Moors et al. 2007;Moors et al. 2009a;Moors et al. 2009b). Recently, we showed that PBDEs affect migration and differentiation of hNPCs via endocrine disruption of cellular thyroid hormone signaling (Schreiber et al. 2009 submitted). In the present study we extend this knowledge by showing that short term PBDE exposure affects intracellular Ca^{2+} signaling in hNPCs.

Materials and Methods

Chemicals. BDE-47 and 6-OH-BDE-47 were synthesized and purified (~99% purity) at the Wallenberg Laboratory of Stockholm University as described by Marsh et al. (1999). All additional chemicals used (unless otherwise noted) were purchased from Sigma–Aldrich (Taufstein, Germany) and were of the highest purity available.

Cell culture. The Normal Human Neural Progenitor cells used in this study were prepared from a single donor (hNPCs, Lonza Verviers SPRL, Belgium). Human NPCs were cultured in proliferation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/ml EGF (Biosource, Karlsruhe, Germany) and 20 ng/ml rhFGF (R&D Systems, Wiesbaden-Nordenstadt, Germany) in a humidified 92.5% air/7.5% CO2 incubator at 37 °C in suspension culture as previously described (Moors et al. 2009;Moors et al. 2007). For Ca^{2+} imaging experiments and patch clamp analysis, we differentiated hNPCS by growth factor withdrawal in differentiation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with N2 (Invitrogen)) and plating onto poly-D-lysine/laminin coated cover glasses (Menzel GmbH & Co KG, Braunschweig, Germany; MatTek, Ashland MA, USA).

Electrophysiology. Experiments were performed at room temperature. During experiments, neurospheres were constantly perfused with artificial saline containing 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM glucose, bubbled with 95% O_2 / 5% CO_2 resulting in a pH of 7.4.

Somatic whole-cell patch-clamp recordings were carried out using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA, USA) coupled to a personal computer via a digidata 1322A interface (Molecular Devices). Data were acquired at 1 to 10 kHz using PClamp 8.2 software (Molecular Devices). Electrodes were pulled from borosilicate glass (Hilgenberg, Waldkappel, Germany) and filled with internal saline containing (in mM): 125 K-Acetate, 10 KCl, 10 HEPES, 4 NaCl, 0.5 EGTA, 1 $MgCl_2$, pH 7.2 (KOH). Electrode resistance was 3-4 MΩ. The performed voltage-step protocol induced large capacitive as well as passive currents and leak subtraction (P/4) was performed to uncover voltage-gated

currents activated by membrane depolarization. Data analysis was performed with Clampfit 8 and IgorPro Software.

Calcium imaging. Ratiometric calcium imaging was performed as described previously (Dingemans et al. 2008). Briefly, cells were loaded with 5-15 µM Fura-2 AM (Molecular Probes; Invitrogen) in saline for 20 min at room temperature. Afterwards the cells were washed with external saline and placed on the stage of a microscope equipped with a TILL Photonics Polychrome IV (TILL Photonics GmbH, Gräfelfing, Germany). Fluorescence was recorded every 0.5-6 sec at 510 nm (excitation wavelengths: 340 and 380 nm, or 357 and 380 nm) with a CCD camera (TILL Photonics GmbH) and the fluorescence ratio (F340/F380 or F356/380) was calculated. Data collection and digital camera and polychromator control were performed by imaging software (TILLvisION, version 4.01). Changes in the fluorescence ratio were analysed using custom-made Excel macros (Microsoft Corp., Redmond, WA, USA) or Igor Pro Software (Wavemetrics, Lake Oswego, OR, USA).

Cells were exposed to 0.2-20 µM BDE-47 or 0.02-20 µM 6-OH-BDE-47 for 20 min, after 5 min baseline recording. Maximum and minimum ratios were determined after 25 min recording by addition of ionomycin (5 µM) and ethylenediamine tetraacetic acid (EDTA, 17 mM) as a control for experimental conditions.

For the experiments under Ca^{2+}-free conditions cells were washed after Fura-2 loading with Ca^{2+}-free external saline (containing 10 µM EDTA) just before the imaging experiments. In specific experiments, where intracellular Ca^{2+} stores were emptied by 10 min incubation with 1 µM thapsigargin (TG) and 1 µM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), cells were exposed to 20 µM BDE-47 or 6-OH-BDE-47 for 15 min.

Receptor agonists were either bath-applied or puff-applied using a Picospritzer II (General Valve/Parker Hanifin, Flein/Heilbronn, Germany) coupled to standard micropipettes with a tip diameter of around 1.5 µm (Hilgenberg, Waldkappel, Germany) placed at a distance of approximately 10–20 µm above the cell layer. Exposure to receptor antagonists was performed by bath application during the whole recording time.

LDH assay. Cell viabilitiy was measured using a lactate-dehydrogenase (LDH) assay (CytoTox-One, Promega) as previously described (Moors et al. 2009a). Briefly, supernatants from cells that were exposed to 0.02-20 µM BDE-47 and 6-OH-BDE-47 for 20 minutes were collected and incubated 1:1 with the CytoTox-One reagent for 4 h prior to detection of fluorescence (Ex 540 nm/Em 590 nm). Complete lysis of cells with the included lysis buffer for 2 h at room temperature serves as positive control.

Data analysis and statistics. To determine effects on $[Ca^{2+}]_i$, the fluorescence ratio of each cell under baseline conditions was normalized. An increase in the normalized fluorescence ratio of more than 1.2 (≥ 1.02 in case of glutamate application) was considered as an increase in $[Ca^{2+}]_i$ and was used for further data analysis. All data are presented as mean ± SE from the number of cells indicated. Statistical analyses were performed using GraphPad Prism. We compared continuous data using paired Student's t-test and categorical data were compared using Fisher´s exact and chi-square tests. For multifactor analysis ANOVA in combination with the Bonferroni *post hoc* test was used. A p-value < 0.05 was considered statistically significant.

Results

Electrophysiology. Cells which had migrated out of the neurosphere during differentiation were characterized electrophysiologically by performing whole-cell patch-clamp measurements. Cells had a mean membrane potential of -42.4 ± 10.7 mV, a membrane resistance of 963.8 ±570 MΩ, and a membrane capacitance of 6.4 ± 3.8 pF (n = 11). To reveal the functional expression of voltage-gated ion channels, cells were held in the voltage-clamp mode at -80 mV and then subjected to a rectangular voltage step protocol from -100 to +50 mV in 10 mV increments. In some experiments, cells were additionally hyperpolarized to -110 mV before the voltage-step protocol to remove inactivation of voltage-gated sodium channels. The voltage-step protocol revealed the presence of outward currents (n = 10/11 cells). In two of these cells, we detected fast inward currents. In none of the cells tested, injection of depolarising currents was able to induce action potentials in the current clamp mode (Supplementary Fig. 1).

General characterization of calcium signaling in hNPCs. Calcium imaging experiments were performed with cells which had grown out of the neurosphere during differentiation. Cells did not show spontaneous calcium transients after different differentiation times ranging from 12 hours to 96 hours (n=394 cells on 18 coverslips; Figure 1). To probe for the presence of voltage-gated ion channels, we applied saline containing 80 mM potassium by puff application for 2.5 s to the cells. Neurons are quickly depolarized by the elevated potassium concentration and the subsequent opening of voltage-gated calcium channels then causes calcium influx and fast calcium intracellular transients. Only a minority of cells (3 of 125 cells analysed on 5 coverslips, differentiated for 48 and 96 hours) responded to the application of potassium with a calcium increase (data not shown).

In contrast to this, virtually all cells responded to puff application of ATP (0.5 or 1 mM for 1 s; 1012/1020 cells on 23 coverslips; Figure 1). The same was true for puff application of acetylcholine (0.5 mM for 1 or 2 s; 856/986 cells on 22 coverslips; Fig. 1 A). The calcium increase induced by acetylcholine was largely blocked by atropine (53 cells on 3 coverslips, not shown), confirming that it was due to the activation of muscarinic receptors on the cells. The percentage of cells responding to ATP and acetylcholine as well as the amplitude of the induced calcium transients was independent of the differentiation time (either 12, 24 or 48 hrs in culture, Figure 1B).

Application of glutamate (1 mM; 1-2.5 s) induced calcium increases in the majority of cells (192/211 on 16 coverslips; Fig. 1 A). The amplitudes of the glutamate-induced calcium transients were, however, quite small as compared to those induced by ATP or acetylcholine and at early differentiation time points (12 hours) the number of responding cells was significantly smaller than at later timepoints (Fig 1 B). Therefore, we chose for all other experiments a differentiation time of 24 or 48 hours. In none of the cells studied (0/60 cells on 3 coverslips), application of GABA (1 mM, 2.5 s) induced an increase in intracellular calcium (Fig 1 A).

BDE-47 as well as 6-OH-BDE-47 cause an increase in $[Ca^{2+}]_i$ in human neural progenitor cells. The acute effects of BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 on calcium homeostasis in hNPCs were measured. The vehicle alone (0.1 % DMSO) causes a slight increase in $[Ca^{2+}]_i$ in 26.7 % of the cells (Figure 2 & 3). BDE-47 initiates a concentration-dependent increase in $[Ca^{2+}]_i$ at concentrations $\geq$ 2 µM. Application of 2 and 20 µM result in an increase in 42.2 and 82.9 % of responding cells, respectively (Figure 2 and 3). Exposure of hNPCs to 6-OH-BDE-47 triggers a concentration-dependent increase in the percentage of responding cells with a LOEL of 0.2 µM. Application of 0.2, 2 and 20 µM 6-OH-BDE-47 leads to an increase to 44.8 %, 54.1 % and 100 % of responding cells, respectively (Figures 2 and 3). Besides the number of responding cells, exposure to BDE-47 or 6-OH-BDE-47 also enlargens the amplitude of $[Ca^{2+}]_i$ in a concentration-dependent manner, with a stronger effect of 6-OH-BDE-47 (Figure 3). Whereas the solvent control leads to an increase in $[Ca^{2+}]_i$ with an amplitude of 1.16 ± 0.01, BDE-47 treatment results in significantly higher amplitudes of 1.24 ± 0.03 (2 µM) and 2.11 ± 0.15 (20 µM, Figure 3C). 6-OH-BDE-47 elevates the amplitude significantly at 0.2 µM (1.30 ± 0.05), and with increasing concentrations the amplitude increases to 1.44 ± 0.08 (2 µM) and 5.74 ± 0.45 (20 µM, Figure 3D). Furthermore, 20 µM BDE-47 raises the average amplitude significantly (1.15 ± 0.03) compared to control (1.07 ± 0.00?). In contrast, the hydroxylated form increases the average amplitude at

concentrations ≥ 0.2 µM (1.14 ± 0.01) up to 3.49 ± 0.33 in case of 20 µM (Figure 3C, D). There is also a difference in response time: while the increase in $[Ca^{2+}]_i$ occurrs 3 to 5 minutes after BDE-47 (20 µM) application with multiple peaks, 6-OH-BDE-47 causes an immediate persistent $[Ca^{2+}]_i$ elevation in concentrations ≥ 0.2 µM. Only 20 µM 6-OH-BDE-47 induce a transient initial $[Ca^{2+}]_i$ increase within the first 5 min after application followed by a late (> 10 min) $[Ca^{2+}]_i$ increase. Some single cells show a very high $[Ca^{2+}]_i$ increase without recovering (Figure 2). Furthermore, exposure to 20 µM 6-OH-BDE-47 results in a general elevation of the baseline, which persists over time (Figure 2). This shift is also reflected in a strong increase in the average amplitude (Figure 3).

Induced increases in $[Ca^{2+}]_i$ originate from extracellular and intracellular stores. To investigate the origin of the observed increases in $[Ca^{2+}]_i$, we performed experiments with PBDEs (20 µM) under Ca^{2+}-free conditions. After removal of extracellular Ca^{2+} no increase in $[Ca^{2+}]_i$, is observed after application of the solvent control DMSO (data not shown), while BDE-47 initiates only a few fast transient $[Ca^{2+}]_i$ increases. Compared to BDE-47 exposure in Ca^{2+}-proficient medium, where up to 4 peaks/cell were recorded, only one peak/cell was measured in Ca^{2+}-deficient medium (Figure 4B). Moreover, extracellular Ca^{2+}-deficiency decreases the number of responsive cells from 83 % to 26 % and the amplitude of the $[Ca^{2+}]_i$ increase was significantly smaller (1.16 ± 0.07 in contrast to 1.72 ± 0.14, Figure 4B, C). We observed a remarkable difference to the parent BDE-47 when we treated the cells with 6-OH-BDE-47. The number of responding cells does not change under Ca^{2+}-free conditions (Figure 4C). Moreover, the initial transient and the shift of baseline in $[Ca^{2+}]_i$ is still present, while the late increase is absent (Figure 4A). The average and the maximal amplitude of $[Ca^{2+}]_i$ are significantly higher under physiological Ca^{2+} conditions, 3.16 ± 0.35 and 4.97 ± 0.50 in contrast to 1.48 ± 0.02 and 2.58 ± 0.11 in Ca^{2+}-free medium (Figure 4D). These data indicate that the parent compounds and the hydroxylated metabolites induce $[Ca^{2+}]_i$ influx in hNPCs through different mechanisms.

To identify the intracellular stores responsible for the observed increase in $[Ca^{2+}]_i$, we performed additional Ca^{2+} imaging experiments using neurospheres in which mitochondrial and TG-sensitive ER Ca^{2+} stores were depleted by pretreatment with TG and FCCP, respectively, in Ca^{2+}-free medium. TG and TG/FCCP pretreatment increase $[Ca^{2+}]_i$ transiently, but after several minutes the baseline stabilizes. Exposure of hNPCs to 20 µM BDE-47 or 6-OH-BDE-47 in absence of extracellular Ca^{2+} and presence of TG drops the number of responding cells to 0 or 84 %, respectively and decreases the amplitude of the response significantly to 1.13 ± 0.01 or 1.41 ± 0.02 in comparison to the effects seen after

PBDE exposure only in Ca^{2+}-free medium (Figure 4C, D). However, the 6-OH-BDE-47-induced shift of the baseline still remains in presence of TG, but appears with a delay of several minutes. Interestingly, depletion of mitochondrial and ER Ca^{2+} stores with both TG and FCCP under Ca^{2+}-free conditions eliminates this baseline shift. However, a single transient increase after 5 to 10 min after application remains in 89 % of all cells (Figure 4C, D).

BDE-47 and 6-OH-BDE-47 are not cytotoxic to hNPCs. To investigate if application of BDE-47 or 6-OH-BDE-47 causes acute cytotoxicity in hNPCs, we performed a lactate-dehydrogenase (LDH) assay. Therefore, hNPCs were exposed to different concentrations of BDE-47 or 6-OH-BDE-47 for 20 min, after which the supernatant was collected and LDH release was analysed. An exposure up to 20 µM BDE-47 or 6-OH-BDE-47 did not result in a release of significant amounts of LDH from the cells compared to solvent control (Figure 5), indicating that the observed effects on Ca^{2+} homeostasis are not caused by cytotoxicity.

Discussion

PBDEs administered during rodent brain development alter neurobehavior of the offspring (rev. in Costa and Giordano 2007). Disturbances in long term potentiation (LTP) possibly by altered calcium homeostasis might thereby be one of the underlying mechanisms (Dingemans et al. 2008;Kodavanti and Ward 2005;Xing et al. 2009). We have already shown that a one week exposure with BDE-47 and BDE-99 did not alter hNPC calcium responses towards ATP, acetylcholine (ACh) and glutamate (Schreiber et al. 2009). However, the direct, short term influences of PBDEs on $[Ca^{2+}]_i$ had so far not been investigated in primary human neural cells. Therefore, the aim of the present study was to determine the acute effects of BDE-47 and the hydroxylated metabolite 6-OH-BDE-47 on $[Ca^{2+}]_i$ homeostasis and signaling in hNPCs.

First, we analyzed the basic electrophysiological properties of hNPCs by patch-clamp experiments and measured the calcium influx after application of different neurotransmitters. The data of the patch-clamp experiments concerning the membrane characteristics were in the same ranges as those reported earlier from cells derived from neurospheres (Piper et al. 2000). Similar to the results obtained by Piper et al., the voltage-step protocol revealed the presence of outward currents, most likely representing a mixture of several potassium currents. The neurotransmitters ATP, ACh and glutamate evoke Ca^{2+} transients in hNPCs. The percentage of cells responding to ATP and acetylcholine as well as the amplitude of the induced calcium transients presented here, was independent of the differentiation time (either 12, 24 or 48 hrs

in culture) and application form (bath application or puff-application), whereas the bath application resulted in slightly higher amplitudes due to a longer exposure time. In case of glutamate, the number of responding cells was significantly smaller at early (12 hours) than at later differentiation timepoints (24-48 hours). It is not unlikely that this observation reflects a developmental regulation in Ca^{2+} signaling because modifications of ligand-induced Ca^{2+} responses by age *in vitro* are already described in other neural cell models (Gafni et al. 2004;He and McCarthy 1994;Takeda et al. 1995) and it is known that glutamatergic and GABAergic neurotransmitter receptors are differentially expressed during prenatal development also *in vivo* (Lujan et al. 2005). In contrast, cells did not respond to high extracellular potassium. These results indicate the presence of functional receptors to ATP, ACh, and glutamate while voltage-gated Ca^{2+} channels are either not expressed or not functional.

While it is the most important inhibitory transmitter in the adult CNS, GABA acts depolarizing in neurons of the developing hippocampus and other brain regions (Ben-Ari et al. 2007;Misgeld et al. 1986;Rivera et al. 1999). The opening of $GABA_A$ receptor channels causes outward movement of chloride at this early stage of development. The resulting depolarization then opens voltage-activated calcium channels and results in intracellular calcium transients. The same is true for astrocytes both in early postnatal development as well as in mature brain (Bekar et al. 1999;Bernstein et al. 1996;Butt and Jennings 1994;Kettenmann et al. 1984;Meier et al. 2008;Nilsson et al. 1993). However, in none of the cells grown from the neurosphere application of GABA induced an increase in $[Ca^{2+}]_i$, suggesting the absence of functional GABA receptors. Taken together, these findings show that hNPCs provide a suitable model to investigate the effects of chemicals on $[Ca^{2+}]_i$.

Exposure towards BDE-47 and its hydroxylated metabolite 6-OH-BDE-47 caused Ca^{2+} transients in hNPCs. Thereby, 6-OH-BDE-47 was more potent causing increases in $[Ca^{2+}]_i$ at lower concentrations than the parent compound. This is in agreement with results in the rat pheochromocytoma cell line PC12 (Dingemans et al. 2008). However, the effects caused in hNPCs appear to be stronger than in PC12 cells. Already 2 µM BDE-47 or 0.2 µM 6-OH-BDE-47 cause significant increases in $[Ca^{2+}]_i$ in hNPCs whereas in PC12 cells rather high concentrations (20 µM BDE-47 and 1 µM 6-OH-BDE-47) were needed to evoke similar responses (Dingemanns et al. 2007; Dingemanns et al 2008). In both cell models, 6-OH-BDE-47 instigated an initial transient and a late persistent increase in $[Ca^{2+}]_i$, whereas only in human neurospheres a shift of baseline was oberserved. Effects of PBDEs on Ca^{2+} homeostasis were also seen in SH-SY5Y cells after 1.5 – 24 hours (25.6 µM of the

pentabrominated mixture DE-71; Yu et al. 2008) and in microsomes and mitochondria isolated from different rat brain regions after 20 min (3–10 µg/ml DE-71; Kodavanti and Ward 2005). However, these measurements were carried out by endpoint determination and not in a time kinetic like in the present study. Therefore, the results are difficult to compare.

Not only PBDEs, but also the known developmentally neurotoxic polychlorinated biphenyls (PCBs) disrupt Ca^{2+} homeostasis in cultured neuronal cells and brain preparations (comprehensively reviewed in Mariussen and Fonnum 2006). This interference with Ca^{2+} signaling is responsible for PCB-induced oxidative stress and apoptosis *in vitro*. Due to structural similarities between PCBs and PBDEs, disruption of $[Ca^{2+}]_i$ balance might also contribute to PBDE-induced DNT and the involved target structures might be similar.

Possible origins for increases in $[Ca^{2+}]_i$ are influx of extracellular Ca^{2+} by voltage-gated Ca^{2+}-channels (Catterall 2000) or store-operated Ca^{2+} entry (SOCE) channels (Parekh and Putney, Jr. 2005). An increase in $[Ca^{2+}]_i$ could furthermore originate from intracellular Ca^{2+} stores, such as ER, mitochondria or the nucleus. Furthermore, loss of membrane integrity can result in an unspecific increase in $[Ca^{2+}]_i$. We ruled out this last possibility in the present study because LDH-activity in the media did not increase upon PBDE treatment.

Generally, PCB- and PBDE-induced effects on Ca^{2+} homeostasis were attributed to influx of extracellular Ca^{2+}. In our study, the removal of Ca^{2+} from external saline results in a dramatic decrease in number of cells responding towards BDE-47. For the hydroxylated metabolite 6-OH-BDE-47, removal of extracellular Ca^{2+} causes a decrease in the amplitude of the initial increase in $[Ca^{2+}]_i$ and the complete loss of the late persistent increase indicating that these increases originate completely (late increase) or partly (initial increase) from influx of extracellular Ca^{2+}. Inglefield and Shafer showed that an extracellular calcium entry via L-type voltage-sensitive channels (VSCCs) was required to elicit Ca^{2+} oscillations because the oscillations induced by PCBs were blocked in Ca^{2+} deficient solution or by addition of the VSCC channel blocker nifedipine (Inglefield and Shafer 2000b). Moreover, the observed PCB-induced Ca^{2+} oscillations were attributed to increased excitatory synaptic activity of excitatory glutamate and GABA receptors (Inglefield and Shafer 2000a). As hNPCs do not possess functioning VSCC or GABA receptors, this mechanism is not involved in the increase in $[Ca^{2+}]_i$ after PBDE exposure of hNPCs.

The remaining peaks were completely absent in presence of tapsigargin (TG) an inhibitor of endoplasmic reticulum (ER) Ca^{2+}-ATPase demonstrating that this residual increase in $[Ca^{2+}]_i$

primarily originates from the ER. This is in agreement with the findings in PC12 cells (Dingemans et al. 2008), where the initial increase is also absent after TG treatment.

The principle mechanisms affecting release of Ca^{2+} from ER stores involve activation of inositol 1,4,5-triphosphate receptors (IP3R) and/or ryanodine receptors (RyR). Wong et al. (1997) reported that *ortho*-PCBs enhance ryanodine binding in membrane preparations from rat brain hippocampus, cerebellum, and cortex, and induce a ryanodine-sensitive Ca^{2+} mobilization in rat brain microsomes isolated from cerebral cortex. Pessah and coworkers have studied the ryanodine effect more thoroughly in later studies, and demonstrated that an *ortho*-PCB (PCB 95) interacts with FK506 binding protein (FKBP12) and ryanodine receptor complex (RyR), and sensitizes RyR-mediated Ca^{2+} release (Gafni et al. 2004;Wong et al. 2001). To answer the question if the BDE-47 and 6-OH-BDE-47 induced Ca^{2+} store depletion from the ER was modulated by interaction between the BDEs and the ryanodine receptor, expression and functionality of this receptor in hNPCs were investigated. Microarray expression analysis revealed the mRNA expression of the brain–specific RyR-3 (Moors & Fritsche, unpublished observation). To investigate the functionality of the RyR in hNPCs, neurospheres were stimulated with the direct RyR agonist 4-chloro-m-cresol (CMC) and the receptor sensitizer caffeine. Neither a puff application of 500 µM CMC nor the puff application of 20 mM caffeine elicits an increases in $[Ca^{2+}]_i$ in hNPCs after different differentiation times (data not shown) indicating that the RyR is not involved in PBDE-induced Ca^{2+} release from the ER.

A later study by Inglefield et al. showed that PCBs also induce an immediate release of Ca^{2+} from the ER involving inositol triphosphate (IP3)-sensitive Ca^{2+} channels (Inglefield et al. 2001). Involvement of the IP3 receptor (IP3R) in calcium efflux from the ER after PCB exposure was further supported by the notion that PCB (Arochlor1254, 30 µM) induced intracellular Ca^{2+} increase and subsequent cell death of a catecholaminergic cell line which was blocked by an IP3R antagonist and to a lesser extent an RyR antagonist (Kang et al. 2004). If IP3R is involved in PBDE-induced ER Ca^{2+} release is subject of future investigations.

After depletion of extracellular Ca^{2+} and ER Ca^{2+} stores the shift of baseline caused by 20 µM 6-OH-BDE-47 still remains. Because this shift disappears after co-administration of the mitochondrial uncoupler FCCP, we suggest that these Ca^{2+} currents originate from mitochondrial Ca^{2+} stores. However, in approximately 90% of the cells a small 6-OH-BDE-47-induced increase in $[Ca^{2+}]_i$ remains even after depletion of ER and mitochondrial Ca^{2+} stores. The origin of these currents has to be further elucidated. These data indicate that the

PBDE-induced initial transient $[Ca^{2+}]_i$ increase originates from the ER, whereas the shift of baseline is mainly due to Ca^{2+} release from mitochondria. The late persistent increase in $[Ca^{2+}]_i$ seems to be caused by extracellular Ca^{2+} influx, since it is absent under Ca^{2+}-free conditions. These results are partly in contrast to PC-12 cells where TG and FCCP co-administration abolished all PBDE-induced increases in $[Ca^{2+}]_i$ (Ciccolini et al. 2003;Dingemans et al. 2007). What are the possible underlying mechanism of the PBDE-induced disruption of Ca^{2+} homeostasis in hNPCs? Inhibition of ER and mitochondrial Ca^{2+}-ATPases, mobilisation of $Ca^{2+}$from ER through interactions with IP3 and sigma receptors, or disruption of ER or mitochondrial membranes are conceivable and have to be further investigated.

In summary, exposure to BDE-47 (2 μM) or 6-OH-BDE-47 (0.2 μM) cause an increase in $[Ca^{2+}]_i$ in hNPCs. This increase is mainly due to release of Ca^{2+} from ER and mitochondria. Thus, 6-OH-BDE-47 is at least one order of magnitude more potent than the parent compound BDE-47. PBDEs are found in human fetal blood samples in concentrations up to 460 ng/g lipid (Mazdai et al. 2003). Taken a blood lipid content of 2 mg/dl into account, this equals a concentration of 2 nM in fetal blood. Additionally, it was shown that brain PBDE concentrations can exceed blood levels by a factor of 10 (Staskal et al. 2006a). Considering that approximately 45% of detectable PBDEs are present as metabolites in human fetal blood (Qiu et al. 2009), PBDE-effects on hNPCs in our study are observed at relevant concentrations close to human exposure. Whereas an association between perinatal exposure to PCBs and DNT in humans was drawn from cohort studies (rev. in Winneke et al. 2002), epidemiologic evidence for DNT of PBDEs is missing due to lack of such studies. However, the strong disrupting effect of 6-OH-BDE-47 on Ca^{2+} homeostasis in hNPCs should be taken into account in human PBDE hazard assessment.

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Figure legends

Figure 1: Differentiated NPCs express functional neurotransmitter receptors. (**A**) Representative traces of ratiometric calcium imaging of 24 h differentiated NPCs that were constantly perfused with ringer solution. Arrows show application of neurotransmitters. All substances were applied for 1 s. In case of glutamate and GABA the graph was smoothed by calculating the mean fluorescence of three timepoints. (**B**) Occurrence and amplitudes of ATP, ACh and glutamate-induced Ca^{2+}-increases on different differentiation time points under constant perfusion and after bath application (b.a.). Data are from 3-4 experiments per treatment. Numbers above bars indicate the number of cells used for the data analysis; values shown are mean ± SD. *$p < 0.05$

Figure 2: Increase in $[Ca^{2+}]_i$ in human neurospheres during exposure to BDE-47 or 6-OH-BDE-47. Results are shown as representative traces of normalized F340/F380 ratio, from individual cells exposed to 0.2 to 20 µM BDE-47 (**A**) or 0.02 to 20 µM 6-OH-BDE-47 (**B**) for 20 min, applied at t = 5 min, as indicated. Note the difference in scaling for 20 µM. (**C**) Representative recording with the characteristic initial transient increase, the late persistent increase and the shift of baselin in $[Ca^{2+}]_i$ used in this article.

Figure 3: Occurrence of $[Ca2+]_i$ disturbances and amplitude during exposure to BDE-47 or 6-OH-BDE-47. (A, B) Bars indicate the percentage of cells showing an increase in $[Ca^{2+}]_i$ within 20 min after application (threshold ≥ 1.2). Numbers above the each bar indicate the number of cells using for data analysis. (C, D) Bars indicate average and amplitudes of increase in $[Ca2+]_i$ after application. Data shown are from 3-4 independent experiments (mean ± SE). * Significances compared to control ($p < 0.05$).

Figure 4: Release from intracellular Ca^{2+} stores in human neurospheres during exposure to BDE-47 or 6-OH-BDE-47. Results are shown as representative traces of $[Ca^{2+}]_i$ measurements of individual cells exposed to 20 µM 6-OH-BDE-47 (A) or BDE-47 (B) for 15 min, applied at t = 5 or 15 min. The diagrams show responding cells (C) and the amplitude (D). Data shown are from 3-4 independent experiments (mean ± SE). * Significances compared as indicated ($p < 0.05$).

Figure 5: PBDEs are not cytotoxic to hNPCs after 20 min. Quantification of cytotoxic effects of PBDEs measured by lactate dehydrogenase release. The LDH release was measured 20 after exposure to the indicated concentrations and compared to the max LDH release after cell lysis. All data are mean ± SEM of three independent experiments ($P < 0.05$).

Figure 1

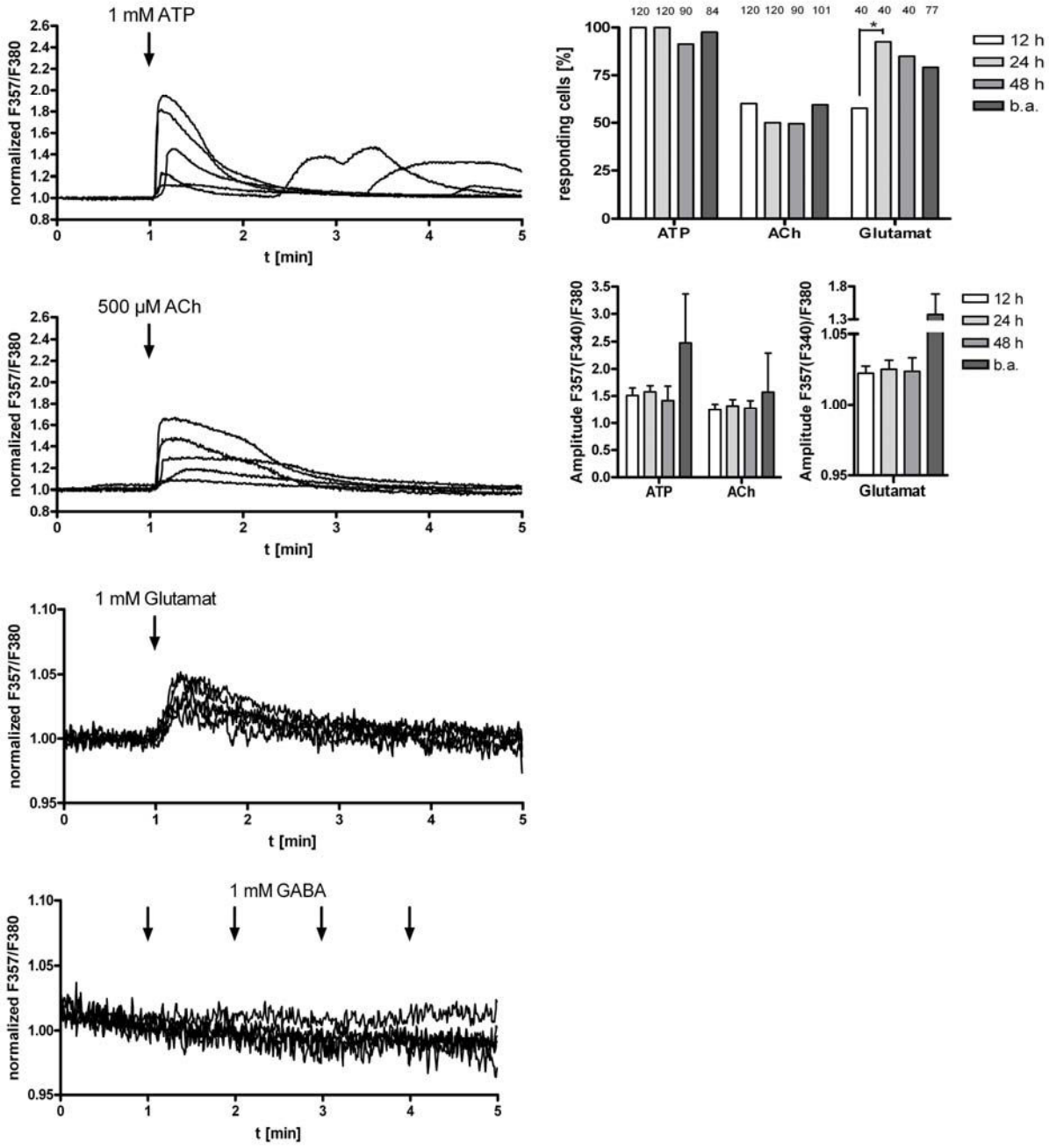
1 mM ATP
normalized F357/F380
t [min]
500 µM ACh
normalized F357/F380
t [min]
1 mM Glutamat
normalized F357/F380
t [min]
1 mM GABA
normalized F357/F380
t [min]
120 120 90 84
120 120 90 101
40 40 40 77
*
responding cells [%]
ATP
ACh
Glutamat
12 h
24 h
48 h
b.a.
Amplitude F357(F340)/F380
ATP
ACh
Amplitude F357(F340)/F380
Glutamat
12 h
24 h
48 h
b.a.

Figure 2

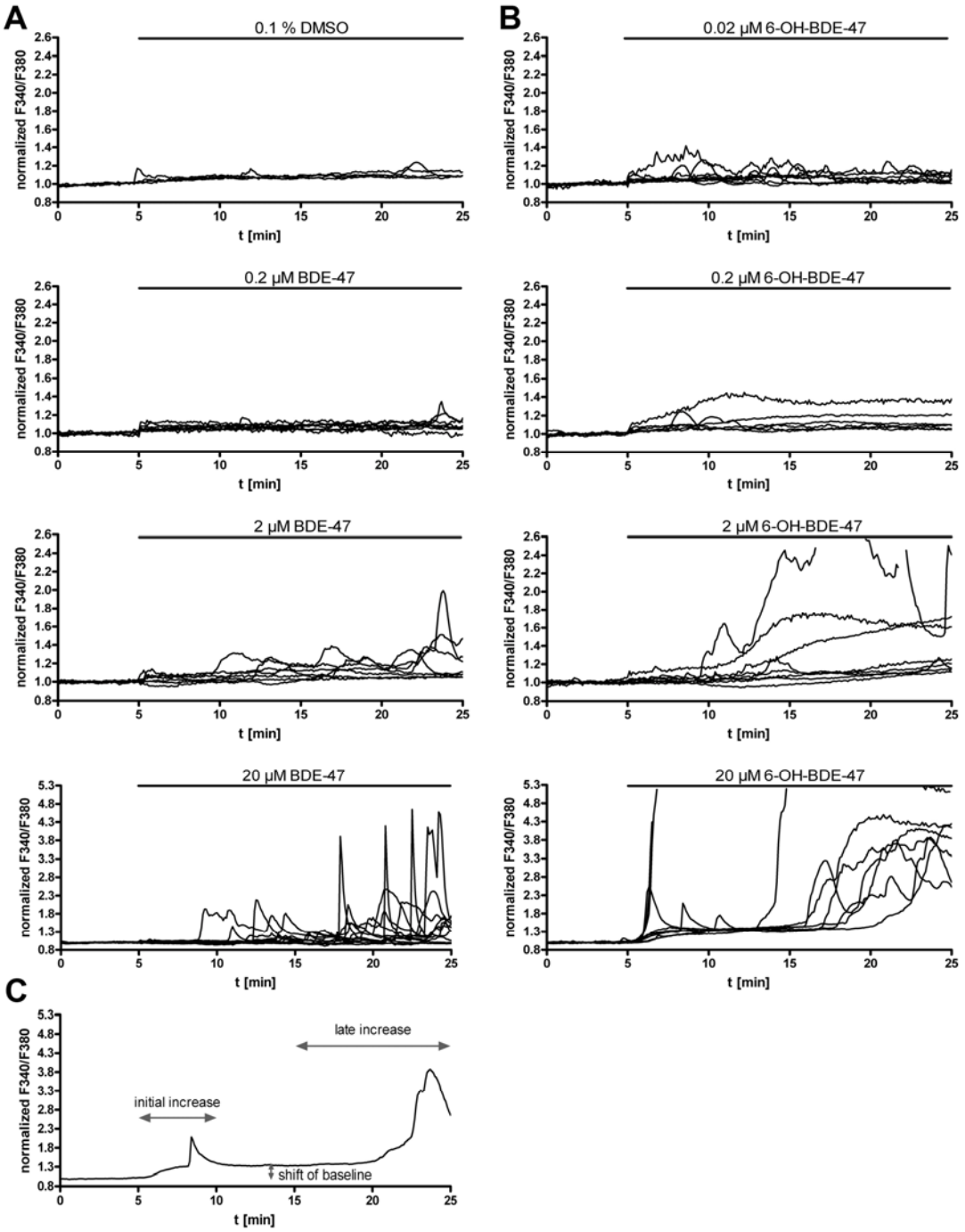
A
0.1 % DMSO
0.2 µM BDE-47
2 µM BDE-47
20 µM BDE-47
B
0.02 µM 6-OH-BDE-47
0.2 µM 6-OH-BDE-47
2 µM 6-OH-BDE-47
20 µM 6-OH-BDE-47
normalized F340/F380
t [min]
C
late increase
initial increase
shift of baseline

Figure 3

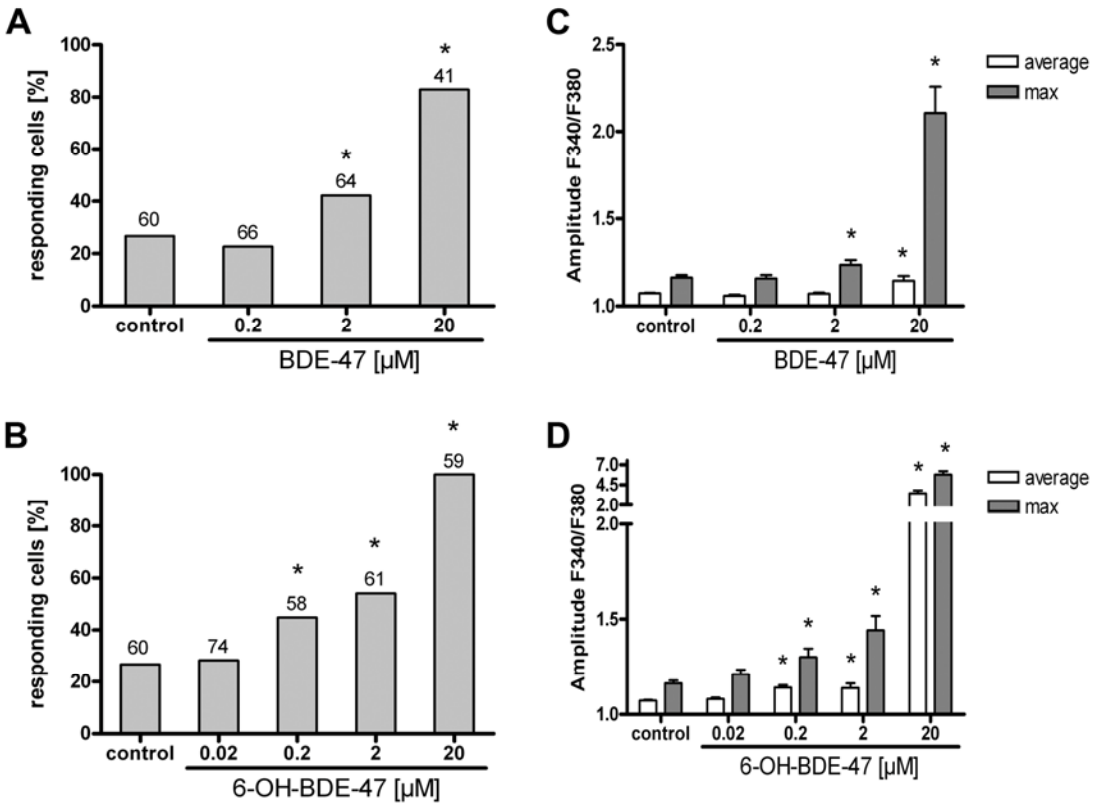

Fig 3: Occurrence of $[Ca^{2+}]_i$ distrubances and amplitude after exposure to BDE-47 or 6-OH-BDE-47. **(A, B)** Bars indicate the percentage of cells showing an increase in $[Ca^{2+}]_i$ within 20 min after application (threshold ≥ 1.2). Numbers above the each bar indicate the number of cells using for data analysis. **(C, D)** Bars indicate average and maximal amplitudes of increase in $[Ca^{2+}]_i$ after application. Data shown are from 3-4 independent experiments. * Significances compared to control ($p < 0.05$).

Figure 4

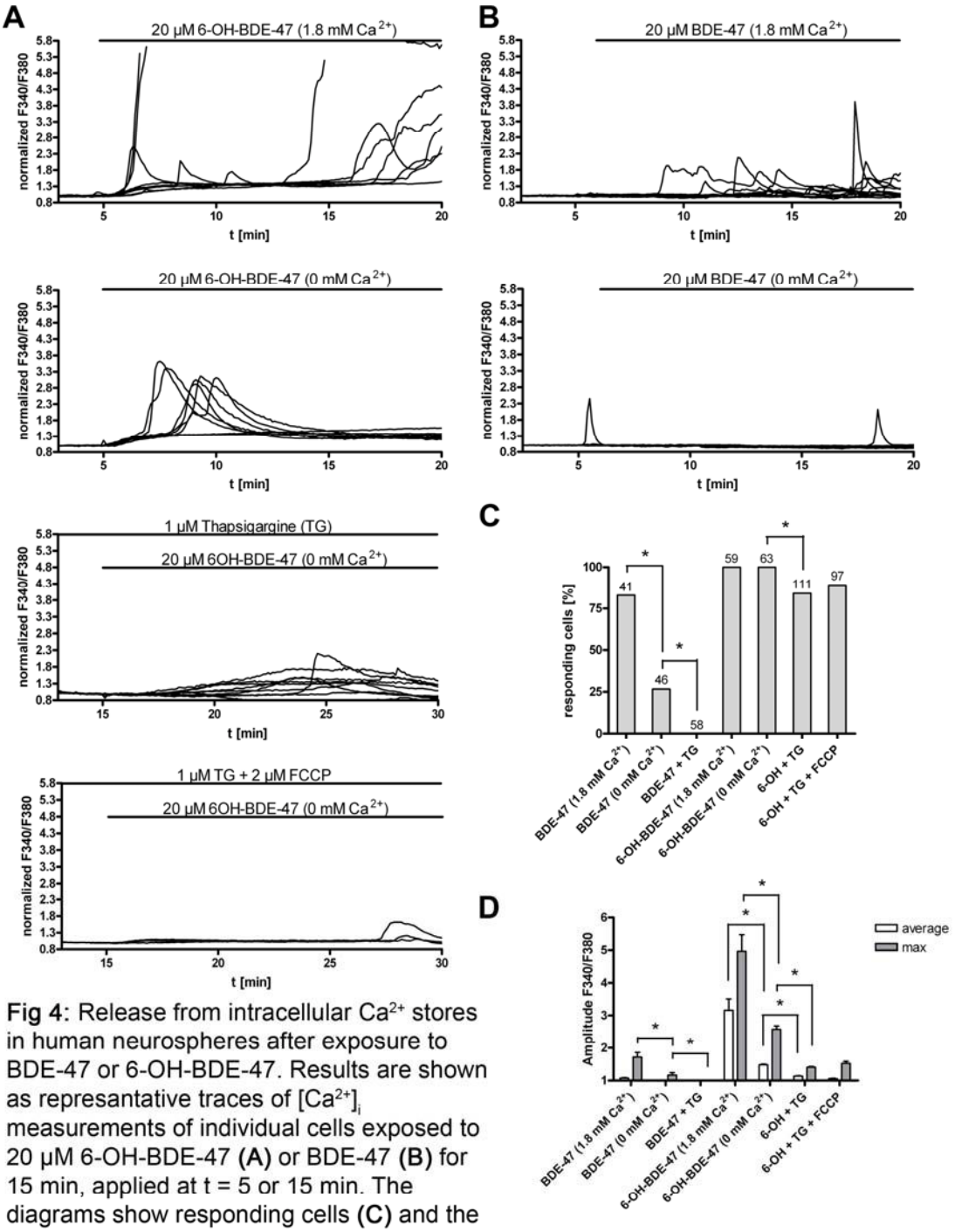

Fig 4: Release from intracellular Ca^{2+} stores in human neurospheres after exposure to BDE-47 or 6-OH-BDE-47. Results are shown as represantative traces of $[Ca^{2+}]_i$ measurements of individual cells exposed to 20 µM 6-OH-BDE-47 **(A)** or BDE-47 **(B)** for 15 min, applied at t = 5 or 15 min. The diagrams show responding cells **(C)** and the amplitude **(D)**. * Significances compared as indicated ($p < 0.05$).

Figure 5

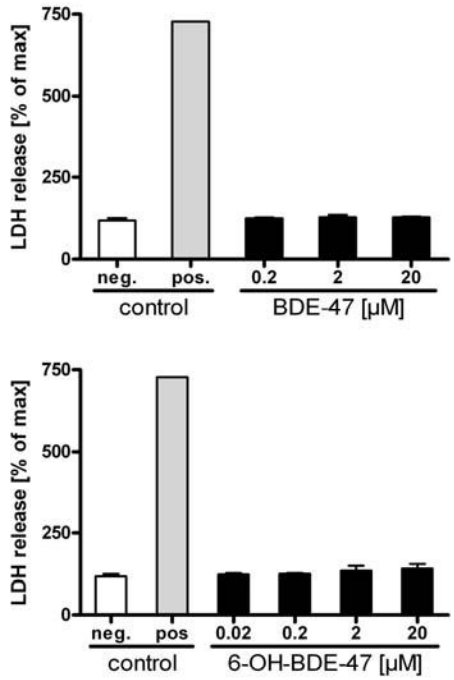

Fig 5: Viability of hNPCs after BDE-47 and 6OH-BDE-47 exposure. The LDH release were measured 20 after exposure to the indicated concentrations and compared to the max. LDH release after cell lysis.

2.6 Green tea catechins inhibit migration and adhesion of neural progenitor cells by blocking β1-integrin ECM interaction

Gassmann K, Zschauer T-C, Ruhl S, Rockel TD, Abel J, Unfried K, Wätjen W, Fritsche E. [Journal of Neurochemistry in Vorbereitung]

Für das sich entwickelnde humane Nervensystem ist die Interaktion zwischen neuralen Progenitorzellen und extrazellulärer Matrix essentiell. Eine Reihe von *in vivo* Studien an Nagern und Vögeln, bei denen entweder die Integrine auf der Zelloberfläche oder das extrazelluläre Matrixprotein Laminin akut blockiert oder komplett ausgeknockt wurden, demonstrieren die Notwendigkeit dieser Interaktion für eine normale neurale Entwicklung.
Es ist bekannt, dass Flavonoide an Zelloberflächenrezeptoren und einzelne Komponenten der EZM binden können und dadurch Zelladhäsion und –migration von verschiedenen Krebszelllinien beeinflussen. In Bezug auf den Einfluss von Flavonoiden auf die Funktion normaler untransformierter Zellen vor allem während der neuralen Entwicklung ist demgegenüber so gut wie nichts beschrieben. Flavonoiden wird eine positive Wirkung auf die Gesundheit zugeschrieben. Aufgrund dessen werden sie vermehrt als Nahrungsergänzungsmittel angeboten. Diese beinhalten die entsprechenden Flavonoide in Reinform und eine kontrollierte Einnahme ist, auch wegen der rezeptfreien Verfügbarkeit, nicht gegeben. Die vom jeweiligen Hersteller empfohlenen Tagesdosen von zumeist 500 mg bis 1 g sind so hoch, dass Plasmakonzentrationen im Bereich von 10 µM erreicht werden können.
Die vorliegende Studie zeigt, dass besonders Grünteeflavonoide (EGCG, EGC, ECG und EC) die humane NPC Migration in Konzentrationen schon ab 2 µM inhibieren. Die verwendeten Flavonoide haben dabei keinen Effekt auf die Zellviabilität und –proliferation. Die beiden Gallat-enthaltenen Grünteeflavonoide (EGCG & ECG) stören die Migration über eine Verringerung der Adhäsion an die EZM, während EGC und EC die Migration wahrscheinlich über alternative Mechanismen beeinflussen. Weiterführende mechanistische Untersuchungen mit blockierenden Antikörpern/Peptiden zeigten, dass eine Störung der Interaktion zwischen der EZM und dem β1-Integrin durch Bindung von EGCG und ECG an Laminin den beschriebenen Effekten zu Grunde liegt. Diese Daten führen zu der Schlussfolgerung, dass die Einnahme von Flavonoiden als Nahrungsergänzungsmittel kritisch hinterfragt werden sollte und dringend weitere Untersuchungen zum toxischen Potential von Flavonoiden nötig sind.

Die Verfasserin der Dissertation führte alle erforderlichen Experimente zu den Endpunkten Viabilität, Proliferation, Migration und einen Großteil der Versuche zur Aufklärung des Mechanismus selbstständig durch. Bei der Etablierung und Durchführung der Expressionsanalysen, Adhäsions- und siRNA-Experimente wurde sie von dem von ihr betreuten Diplomanden Tim-Christian Zschauer unterstützt.

Green tea catechins inhibit migration and adhesion of neural progenitor cells by blocking β1-integrin ECM interaction

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Abbreviations

67LR	67kD laminin receptor
CAM	cellular adhesion molecule
CNS	central nervous system
ECM	extracellular matrix
EC	epicatechin
EC_{50}	concentration that evokes 50% of the effect
ECG	epicatechingallate
ECL	enhanced chemiluminescence
EGC	epigallocatechin
EGCG	epigallocatechingallate
EGF	epidermal growth factor
FGF	fibroblast growth factor
GW	gestation week
H	hesperetin
hESCs	human embryonic stem cells
HMEC-1	human microvascular endothelial cell line 1
K	kaempherol
NPCs	neural progenitor cells
PDL	poly(D)lysin
Q	quercetine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SMCs	smooth muscle cells
siRNA	small interfering RNA
XH	xanthohumol

Introduction

Studies on a wide variety of cells have shown that the extracellular matrix (ECM) governs myriad cell functions including mitosis, apoptosis, migration, cytoplasmic signalling, and transcription (Bokel and Brown 2002;De Arcangelis and Georges-Labouesse 2000;Giancotti and Ruoslahti 1999;Howe et al. 1998;Stupack and Cheresh 2002). Recently, several groups have shown that ECM also regulates rodent and human neural progenitor cell (NPC) function. Proliferation of mouse neuroepithelial cells and migration of mouse cerebellar neural precursor cells *in vitro* (Drago et al. 1991;Kearns et al. 2003), as well as migration of neural precursors through the mouse rostral migratory stream *in vivo* (Belvindrah et al. 2007;Murase and Horwitz 2002), are influenced by the ECM. These effects are not limited to rodents. A publication by Flanagan et al. (2006) compared the effect of laminin on human and mouse NPC development and demonstrated that laminin matrices enhanced NPC migration, expansion, differentiation and neurite elongation both in human and mouse NPCs. Similar results were shown recently by Ma and colleagues in hESCs derived NPCs (Ma et al. 2008).

ECM effects on mouse and human neural precursor cells have been linked to activation of adhesion molecules on the cell surface (Campos et al. 2004;Flanagan et al. 2006;Jacques et al. 1998;Tate et al. 2004). These proteins are transmembrane receptors and most of them belong to one of the following three subclasses: the Ig (immunoglobulin) superfamily, the integrins and the cadherins. It is suggested that a disrupted interaction between these cell surface proteins and the ECM can cause a disturbed brain development. For example, it has been hypothesized that ethanol acts via disruption of L1 mediated cell adhesion as developmental neurotoxicant (rev. in Bearer 2001). Acute inhibition experiments in avian embryos have documented important roles for integrins in migration of the neural crest (Tucker 2004) and in mice, genetic ablation of β1 integrins results in severe perturbations of the peripheral nervous system, including failure of normal nerve arborization, delay in Schwann cell migration, and defective neuromuscular junction differentiation (Pietri et al. 2004). Recent investigations of mice that lack β1-integrins in neural crest development revealed alterations of the radial glial scaffold, resulting in pathological changes also observed in human cortical dysplasias (Graus-Porta et al. 2001). In addition to direct effects on migration, it has been shown that absence of specific integrin heterodimers compromises NPC survival, proliferation, and differentiation (Campos et al. 2004;Feltri et al. 2002;Haack and Hynes 2001;Hall et al. 2008;Jacques et al. 1998;Sakaguchi and Radke 1996;Tomaselli et al. 1993;Weaver et al. 1995;Werner et al. 2000).

It is known that flavonoids especially green tea catechins can bind to cell surface receptors (Kawai et al. 2004;Tachibana et al. 2004) and to ECM components like laminin thus inhibiting

cell adhesion and migration of different transformed cell lines (Benelli et al. 2002;Bracke et al. 1987;Liu et al. 2001;Suzuki and Isemura 2001). But so far there are only limited data concerning the effect of flavonoids on normal cell function especially during neural development.

Flavonoids are a large family of plant-derived polyphenolic compounds widely distributed in fruits and vegetables and therefore regularly consumed in the human diet (Scalbert et al. 2005). Especially Green tea contains many polyphenols such as flavanols (catechins), flavonols, flavandiols, and phenolic acid. The major green tea catechins are epigallocatechingallate (EGCG), epigallocatechin (EGC), epicatechingallate (ECG), and epicatechin (EC) (Graham 1992). A number of physiological benefits have been attributed to these flavonoids, including protection from cardiovascular disease and cancer. Due to these beneficial effects, these compounds are increasingly used as dietary supplements in functional food. Tablets or capsules containing 300 mg quercetin, 1 g citrus flavonoids, or 20 mg resveratrol, with suggested use of 1-6 tablets or capsules per day, are commonly found on the internet. This would result in intakes up to 100 times higher than the common intakes in a Western diet (Mennen et al. 2005) resulting in plasma levels of 0.3-7.5 μM in humans (Ullmann et al. 2003). The potential of such high doses for toxicity, however, is an understudied field of research.

Therefore, the aim of this study was to assess the effects of different flavonoids on human neural development *in vitro*. The model system we used consists of human neural progenitor cells (NPCs) which grow as neurospheres in culture. Neurospheres are an established model system for studying human brain development *in vitro* because they are three dimensional system-based structures, are self-organized and have the potential to differentiate into the three major cell types of the brain: Neurons, astrocytes and oligodendrocytes. We have already shown that known neurodevelopmental toxicants cause effects on viability, migration, differentation and proliferation in our modell system (Fritsche et al. 2005;Moors et al. 2007;Moors et al. 2009). Hence we had a suitable modell system to evaluate the effect of flavonoid-rich food supplements on human neural development. We found that all tested green tea catechins inhibit migration without changes in cell viabilty and proliferation. In case of gallate containing green tea flavonoids a disturbed adhesion capacity seems to be the cause for the defective migration. Mechanistic studies with different kinds of cell adhesion molecule blocking peptides and antibodys showed that EGCG and ECG block the interaction between β1-integrin and of the ECM by binding to laminin.

Material and Methods

Chemicals

All chemicals used (unless otherwise noted) were purchased from Sigma–Aldrich (Munich, Germany) and were of the highest purity available.

Cell culture and flavonoid treatment

The normal human neural progenitor cells (NPCs) used in this study were prepared from three different individuals (Lonza Verviers SPRL, Belgium). NPCs were cultured in proliferation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with B27 (Invitrogen GmBH, Karlsruhe, Germany), 20 ng/ml EGF (Biosource, Karlsruhe, Germany), 20 ng/ml rhFGF (R&D Systems, Wiesbaden-Nordenstadt, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified 92.5% air/7.5% CO_2 incubator at 37 °C in suspension culture as previously described (Moors et al. 2007). Differentiation was initiated by growth factor withdrawal in differentiation medium (Dulbecco´s modified Eagle medium and Hams F12 (3:1) supplemented with N2 (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin) and plated onto poly-D-lysine/laminin coated chamber slides or multiwell-plates. For coating, multiwell plates or chamber slides were coated first with poly-D-lysine (0,1 mg/ml) for 2 hours at 37°C, washed one time with sterile water and then coated with laminin again for 2 hours at 37°C. Laminin concentration was 5 µg/ml for all experiments except the titration experiment. After the laminin coating surfaces was washed again with sterile water and were then stored covered with sterile PBS for at most 1 week at 4 °C. For treatment of neurospheres, flavonoids were solved in DMSO as stock solutions and stored at -20°C . Prior exposure stock solutions were diluted 1:1000 in proliferation or differentiation medium.

Antibodies and peptides used in function-blocking studies

The anti-integrin β1 blocking antibody and the respective isotype control used in this study were from BD, Pharmingen. The anti-integrin α6 blocking antibody and the respective isotype control used in this study were from AbD Serotec, Oxford, United Kingdom. The sequence of the RGD and RAD peptides (Merck KgaA, Darmstadt, Germany) used were: GRGDS (RGD peptide) and GRADSP (RAD peptide). They were solved as 50 mM stock solutions in 5% acidic acid and stored at -20°C according manufacturer's guidline. In function-blocking studies neurospheres or dissociated NPCs were pretreated for 1h in differentiation medium with the respective peptide or antibody concentrations and were then plated onto poly-D-

lysine/laminin coated chamber slides or multiwell-plates under further antibody/peptide-treatment.

Cell Viability, Proliferation and Migration Assay

Cell viability was measured using CellTiter-Blue Assay (Promega, Mannheim, Germany) according to the manufacturer's description with a fluorimeter (Fluoroscan Ascent, Labsystems, Frankfurt, Germany) as described in Moors et al. 2007. We used staurosporine, a potent inducer of the intrinsic apoptotic pathway, as positive control. Proliferation was assessed with a combination of CellTiter-Blue Assay which measures mitochondrial reductase activity and microscopical determination of sphere diameter (Moors et al. 2009). Migration analysis was performed after 48 hours differentiation as previously described (Moors et al. 2007).

Adhesion Assay

Neurospheres were dissociated, resuspended in differentiation medium and plated onto PDL/laminin coated 96-well plates in presence or absence of flavonoids or antibodies. After 1h incubation at 37°C in a humidified 92.5% air/7.5% CO_2 incubator the medium consisting non-adherent cells was aspirated. A washing step with PBS + Ca^{2+}/Mg^{2+} (Gibco, Invitrogen) followed to remove remaining non-adherent cells. The number of remaining cells was quantified using the CellTiter-Blue Assay according to the manufacturer's description as described in the cell viability section. Each experiment was done as a six-fold determination. For the adhesion assays with β1-integrin antibody treatment, spheres were dissociated and preincubated for one hour with the respective antibody concentrations in differentiation medium prior plating onto the PDL/laminin coated surface of the 96-well plates.

Real-time PCR

RNA of proliferating or 48h differentiated neurospheres was prepared with the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Reverse-transcriptase-polymerase chain reaction was performed as previously described (Fritsche et al. 2005). Real-time PCR was performed by using the LightCycler instrumentation (Roche). The PCR mix consisted of 1/10 volume of QuantiTect SYBR green PCR Master Mix (Qiagen, Hilden, Germany), 0.5 µM solutions of each primer, 2 µl of cDNA, and DEPC (diethylpyrocarbonat)-treated H_2O in a final volume of 20 µl. The application started with an initial incubation step of 15 min at 95°C to activate the DNA polymerase. The conditions for PCR amplifications were 40 cycles of 15 sec at 94°C for denaturation, 25 sec of primer annealing, 30 sec at 72°C for elongation, and 2 sec at 72°C for fluorescence detection. Intron spanning primers were designed using PRIMER 3 Software to reduce

genomic amplification. Primer sequences and annealing temperatures were the following: **ITGA1**: (F)AACGAGGCACAATTCTGGAC (R)CGGTAGCCCATCTTTGGATA (annealing temp. 50°C) **ITGA2**: (F)AACGGGTGTGTGTTCTGACA (R)CTTTGTGGGGCCTATATCCA (annealing temp. 50°C) **ITGA3**: (F)AACTGTGGAGGATGTAGGAAGC (R)AAGAGTGAGGTTGAGAGGGTTG (annealing temp.: 50°C); **ITGA5**: (F)AACTCTCGCCGATTCACATC (R)TTCTGCTCCCCAAACACTTC (annealing temp.: 56°C); **ITGA6**: (F)TTTATCGGTCTCGGGAGTTG (R)GGCCACTGAATGTTCAAGGT (annealing temp.: 56°C); **ITGA7**: (F)CTGCCTGTCCAATGAGAATG (R)AGAAGAGTTGCTGGGGAATG (annealing temp.: 60°C); **ITGA10**: (F)GACCCCAGAACAAGGAAACA (R)CACAGCCACATCAGCAAAAC (annealing temp.: 56°C); **ITGAV**: (F)TGGAGTTGCTCAGTGCTTGA (R)AATGCCCCAGGTGACATTAG (annealing temp.: 60°C); **ITGB1**: (F)TCTGCGGACAGTGTGTTTGT (R)CGTTGCTGGCTTCACAAGTA (annealing temp.: 60°C); **ITGB2**: (F)TGCTGAAGCTGACCAACAAC (R)GTCATCAGTGGCAAACACCA (annealing temp.: 56°C); **ITGB3**: (F)CTCGAAAACCCCTGCTATGA (R)AAACACCAGCAAGTGGGATG (annealing temp.: 56°C); **ITGB4**: (F)TCTGGCCTTCAATGTCGTCT (R)CAATAAGCAGCATCCGGTTC (annealing temp.: 56°C); **ITGB5**: (F)TCTACAAAACCGCCAAGGAC (R)GATCGCTCGCTCTGAAACTT (annealing temp.: 54°C); **ITGB8**: (F)CAGCCTGGGTGTTTTCACTT (R)ATCAACTGAGCAGCCTTTGC (annealing temp.: 60°C); **β-ACT**: (F)CCCCAGGCACCAGGGCGTGAT (R)GGTCATCTTCTCGCGGTTGGCCTTGGGGT (annealing temp.: 60°C). For qualitative determinations fragments were separated on a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light. For quantitative measurements of β-integrin mRNA expression levels were normalized to the expression of β-actin. Gene expression was evaluated using the C_t value from each sample. The method of calculation is based on the method of the $\Delta\Delta C_t$ (Livak and Schmittgen 2001). The quantification of the results was obtained while successively calculating: ΔC_t = average C_t value (triplicate) of the target gene - average C_t value (triplicate) of gene control (beta-actin) and; $\Delta\Delta C_t$ = ΔC_t of the transfected - ΔC_t of the untransfected control. For determining absolute copy numbers amplified cDNA was used to generate standard curves.

siRNA and transfection

Two neurospheres (0.8 mm) per well of a 24-well plate were dissociated and resuspended in 500 µl differentiation medium. Cells were transfected with the HiPerFect transfection reagent from Qiagen (Qiagen, Hilden, Germany) in a concentration of 6 µl/well and a specific β1-integrin silencing siRNA or a not-silencing control siRNA from Santa Cruz (Santa Cruz Biotechnology, Heidelberg, Germany) at a final concentration of 1 µM siRNA/well. Cells

treated with HiPerFect alone were used as additional negative control. Cells were incubated for 5h in a humidified 92.5% air/7.5% CO_2 incubator at 37 °C. Transfection was stopped by adding 2 ml proliferation medium/well. After 24h, 48h, 72h and 96h under these proliferative conditions cells were harvested to determine transfection efficiency by real time PCR. 63h and 87h post transfection the ability to adhere was measured by adhesion assay.

Statistics

All results are mean and SEM of at least three independent experiments. We used analysis of variance combined with the Bonferroni post hoc test for multifactor analyses (concentration and time effects), and the Student's *t*-test for two-group comparisons (treatment vs. control) The significance value was set at $p< 0.05$. The EC_{50} were determined with GraphPad Prism.

Results

Effects of flavonoids on NPC viability and proliferation

To determine the cytotoxicity of the flavonoids, spheres were incubated for 2 days with concentrations from 1-10 µM of the respective flavonoid under differentiating conditions. Measurement of mitochondrial activity by CellTiter-Blue Assay did not show any significant decrease in viability compared to the DMSO controls. However, 1 µM staurosporine (positive control) decreased cell viability after 24 hours (Suppl Mat. Fig. 1). Thus, the tested flavonoids did not cause cytotoxicity of hNPC. For assessment of hNPC proliferation under flavonoid exposure, neurospheres were cultured with and without EGCG and EGC (1 – 10 µM) for two weeks. To notice an antiproliferative as well as a proliferative effect, experiments with and without EGF and FGF as proliferative stimuli in combination with these flavonoids were performed. Increase in cell number was determined by measuring in sphere diameter and metabolic activity (Moors et al. 2009). The results show that - in comparison to the negative control without mitogens and the positive control with mitogens – flavonoids did not influence hNPC proliferation significantly over time (Suppl. Mat. Fig. 2 A & B).

Green tea flavonoids inhibit hNPC migration

Human neurospheres were cultivated under differentiating conditions with or without different concentrations of flavonoids for 48 hours. To measure migration, the distance between the sphere edge and the furthest migrated cells was determined 48 hours after plating. Whereas neurospheres of the solvent control group attached to the PDL/laminin coated surface and NPCs migrated 578.6 ± 33.0 µm radially out of the sphere, exposure with especially the

green tea flavonoids EGCG, EGC, ECG and EC resulted in a reduced migration distance at low concentrations and at concentrations higher than 1 µM to an additionally disturbed chain like migration pattern (Figure 1; video in Suppl Mat.). For example, EGCG treated hNPCs wandered 404.7 ± 85.4 µm (83.4 ± 5.1 % of control) at 1 µM and 21.3 ± 12.7 µm (4.0 ± 2.1 % of control) at 5 µM, at 10 µM there was no migration out of the neurosphere detectable. All green tea catechins showed a similiar dose-dependency with EC_{50} of 1.7 µM for EGCG, 2.0 µM for EGC, 1.9 µM for ECG and 4.3 µM for EC. In addition quercetin and xanthohumol inhibited hNPC migration significantly in the hightest dose used (10 µM) which resulted in EC_{50} of 4.7 µM in case of quercetin and an EC_{50} > 10 µM for xanthohumol. The inhibitory effect of quercetin was accompanied by a change in cell morphology and the effect of xanthohumol on migration was very weak, therefore we decided to only investigate the effects of the green tea flavonoids further.

Green tea flavonoids interact with ECM and not directly with intracellular signaling pathways

To explore the mechanism behind the inhibition of migration due to flavonoid exposure, we tested the possibility that the antimigratory effect is connected with the well-described antioxidative effect of flavonoids. Therefore we treated neurospheres for 48 hours under differentiating conditions with two other known antioxidants vitamin C and trolox, a water-soluable vitamin e analogon. Neither vitamin C nor trolox had the abiltity to block migration in concentrations up to 100 µM (Suppl. Mat. Fig. 3).

It is well-known that catechins can bind to the ECM protein laminin (Bracke et al. 1987). Therefore we arranged an experiment design to test the possibility that the green tea flavonoids interact with the PDL/laminin matrix rather than directly with intercellular signaling pathways. We performed three different exposure scenarios: (i) EGCG exposure during differention for 48 hours (control experiment), (ii) 24 hours pretreatment of neurospheres followed by 48 hours differentiation without EGCG and (iii) 24 hours pretreatment of the PDL/laminin coated chamber slides without neurospheres followed by 48 hours differentiation without EGCG. The microscopic pictures in Fig. 2A clearly show that EGCG interacted with the ECM resulting in a disturbed migration at concentrations higher than 1 µM. Fig. 2B shows that there is a connection between laminin concentration and migration ability upon EGCG treatment. Under standard conditions with a 5 µg/ml laminin coating, EGCG reduced the migration dose-dependently at 2 µM (69.0% ± 8.1% of sc), 5 µM (23.0% ± 4.7 of sc) and 10 µM (11.2% ± 5.6 of sc). With rising laminin concentrations the migration distance in the solvent control remained constant while the EGCG effect on migration was decreasing until at 100 µg/ml laminin coating even 10 µM EGCG had no significant effect on migration distance anymore.

Green tea flavonoids affect the adhesion of hNPCs

From our data we hypothesise that green tea flavonoids inhibit the adhesion of hNPCs thus causing the observed migration distubances. To address this question an adhesion assay was established. Neurospheres were dissociated and plated in 96-well plates in presence of the different flavonoids. After 1 h adhesion time loose cells were removed and the number of adherent cells was quantified by CellTiter-Blue Assay. Fig. 3 shows that EGCG, ECG and EGC inihibited cell adhesion with gallate-containing green tea catechins exerting the strongest effect (EGCG > ECG > EGC). Again similar to the migration experiment quercetin had a small but significant effect on adhesion in doses of 5 µM and 10 µM.

hNPC migration is dependent on β1-integrin

Cell adhesion to the ECM depends on specific cell adhesion molecules, mainly on integrins and cellular adhesion molecules (CAMs). RT-PCR analysis revealed that neurospheres express integrin α5-7, αV, β1, β4 (weakly), β5 and β8 on the mRNA level. Integrin α1-3, α10, β2 and β3 mRNAs were not detectable after 40 cycles (data not shown). Integrins are heterodimers that consists of one α- and one β-subunit. There are known 18 α- and 8 β-subunits which can dimerize in different combinations. Some of these dimers can bind to laminin (Humphries et al. 2006). Regarding to this information, there are three possible-heterodimers expressed in hNPCs that could possibly take part in cell adhesion to laminin: α7β1, α6β1 and α6β4. To mimick the effect of the green tea catchins on hNPC migration and adhesion, we treated neurospheres with different kinds of cell adhesion molecule blocking antibodies or peptides. As shown in Fig. 4 A & B neither the RGD peptide (10 – 50 µM) which inhibits binding mainly to fibronectin nor a specific α6-blocking antibody (1 – 10 µg/ml) had an effect on hNPC migration after 48 hours differentation. But as shown in Fig 4 C & D a functional blocking β1-intgrin antibody could mimick the green tea flavonoid effect. At low concentrations of 1 µM – 10 µM the antibody exposure resulted in a diminished migration distance whereas treatment with an isotype control antibody had no effect on migration. Untreated hNPCs migrated over a distance of 785.4 µm ± 45.3 µm, 1 µg/ml β1 antibody reduced the migration distance to 55.1 % ± 15.0 % of control, 4 µg/ml to 43.9 % ± 9.9 % and 10 µg/ml to 51.3 % ± 12.5 %. Higher antibody concentrations of 20 µg/ml and 50 µg/ml resulted in an additional disturbed migration similar to the green tea flavonoid effect at concentrations higher than 1 µM (Fig. 4 C).

hNPC adhesion can be blocked by β1-antibody

To address the question if the β1-integrin antibody does not only inhibit migration but additionally influence adhesion an adhesion assay and a siRNA knockdown experiment were performed. Fig. 5 A shows that the β1-integrin antibody reduced the adhesion at the highest

concentration of 50 µg/ml significantly to 50.3% ± 9.4% of control. Transfection with a specific β1-integrin siRNA resulted in a reduced β1-integrin mRNA expression 24h, 48h, 72h and 96 hours post transfection. The strongest knockdown efficiency was observed after 48 hours with a reduction of 63.2% ± 6% in comparison to cells not treated with siRNA. After 63 hours and 87 hours post transfection, adhesion assays were carried out but no change was observed in the number of adherent cells at both timepoints.

Co-treatment with β1-integrin antibody and EGCG has an additive effect on hNPC migration

Fig. 5 shows the results of the co-treatment with 2 µg/ml β1-integrin antibody or an isotype control antibody with rising concentrations of EGCG. The migration distance of the solvent control without antibodies was 790.4 µm. The antibody treatment alone had only mild effects on migration and reduced the migration distance to 63.4 % ± 7.6 % of control. Co-treatment with 1 µM EGCG led to a strong and significant reduction of migration distance to 30.1% ± 14.1% of control whereas EGCG in a dose of 1 µM alone had only a slight and not significant effect on migration. Similar was seen with 2 µM EGCG, cells with flavonoid exposure alone migrated 53.3 % ± 16.9 % of control but in combination with the β1-integrin antibody migration was further reduced to 12.2 % ± 7.3 %. The isotype-control antibody had no additional effect on hNPC migration. The addition of rising concentrations of β1-integrin antibody to the constant concentration of 2 µM EGCG led to similar results (data not shown).

Discussion

Many functions of NPCs are determined by surface adhesion receptors involved in interactions between cells and ECM proteins (rev. in Campos et al. 2004;rev. in Moore and Larue 2004). In the present study we investigated if flavonoids, especially green tea catechins, have an influence on hNPC migration and adhesion. Our results indicated that all green tea catechins and additionally quercetin and xanthohumol inhibit hNPC migration without changes in cell viability or proliferation at the tested concentrations. The effect of the green tea catechins seems to be mediated by the ECM because pre-treatment of the PDL/laminin coated surfaces blocked the migration but the pre-treatment of the neurospheres had no effect on the migration distance. Additionally rising concentrations of laminin in the coating could dimish the inhibitory effect. An adhesion assay revealed that in case of gallate containing green tea flavonoids a disturbed adhesion capacity seems to be the cause for the defective migration.

This is in concert with published data of various cell lines showing for example that xanthohumol has an antiinvasive effect in human breast cancer cell lines at 5 µM

(Vanhoecke et al. 2005) and inhibits HMEC-1 migration in nanomolar concentrations (C. Gerhäuser personal communications). Several studies have shown that a gallate group in catechins plays an important role in their biological activities including inhibition of matrix metalloproteinases (Sazuka et al. 1997), apoptosis induction (Hibasami et al. 1996;Saeki et al. 2000), inhibition of telomerase (Naasani et al. 1998) and also for the binding to ECM proteins thereby influencing cell adhesion (Cheng et al. 2005;Lo et al. 2007;Suzuki and Isemura 2001). EGCG and ECG were found to inhibit adhesion of mouse melanoma B16 cells to laminin while catechin, EC and EGC had no effect. In line with our results the adhesion to laminin pre-treated with EGCG was also impaired, while pre-treatment of the cells with EGCG in the same concentrations had no effect on adhesion (Suzuki and Isemura 2001). Lo et al. 2007 showed that EGCG and ECG but not catechin in concentrations of 20 µM or higher were able to inhibit smooth muscle cell (SMC) adhesion and migration on collagen and laminin via interference with cell-ECM interaction. Pretreatment of laminin with EGCG and ECG (both 25 µM) prevented SMC adhesion to this protein, but in contrast to our results pretreatment of cells reduce adhesion as well. Similar results were obtained by Hung et al. 2005 with fibroblast exposed to 50 µM EGCG on fibronectin and fibrinogen matrices. The differences to our data may be explained by the higher EGCG doses used in the studies resulting in unspecific binding to the cell surface and the different matrix composition. Moreover it is described in literature that EGCG can bind to the 67-kDa laminin surface receptor (67LR) and remains bound even after removal of EGCG from medium (Tachibana et al. 2004). The 67LR binds laminin with high affinity and specificity (rev. in Nelson et al. 2008) and it was already described that a 67LR antibody blocks cell adhesion (Orihuela et al. 2009). Therefore it is possible that the oberservations of Hung and Lo, that pre-treatment of cells is as effective as pre-treatment of the matrix, were caused by a blockage of the 67LR by EGCG. To answer the question if the 67LR plays a role in our system too, we blocked neurospheres with a 67LR-antibody. There was no inhibition of migration in dilutions up to 1:50 (data not shown). Together with the results of the pre-treatment experiment this cell surface receptor seems not to be essentiell in hNPC migration and adhesion.

Based on our data we suggest that EGCG binds to laminin thus blocking the ECM surface for interaction with cellular adhesion molecules. It was already published in 1987 that catechins can bind to laminin and abrogates the effect of laminin on cell morphology and adhesion in virally transformed fetal mouse cells (Bracke et al. 1987) and later confirmed in different cell lines (Lo et al. 2007;Suzuki and Isemura 2001). To answer the question which integrins are involved in the cell-laminin interaction in our modell system RT-PCRs were performed. The results of the mRNA expression analysis are overall in line with in literature described expression data. Independent of the method and the cell source used there is consistent evidence that hNPC express the α subunits 5, 6, 7, V and the β subunits 1, 5 and 8

(Flanagan et al. 2006;Hall et al. 2008;Jacques et al. 1998;Ma et al. 2008). Flanagan and collagues and Ma and collagues also described integrin β4 as expressed on the cell surface whereas Hall and collagues have never seen integrin β4 mRNA expression. This reflects our expression data that refer to a low expression of this integrin (Flanagan et al. 2006;Hall et al. 2008;Ma et al. 2008). Regarding to this there are three possible-heterodimers expressed in our hNPCs that could possibly take part in cell adhesion to laminin: α7β1, α6β1 and α6β4. Two of these heterodimers contain integrin β1 therefore we decided to focus our study on this integrin.

Integrins are not only regarded as main receptors for laminin but also for fibronectin. But our data clearly show that the fibronectin binding integrin heterodimers are not essentiell for hNPC migration on a PDL/laminin matrix. Treatment with RGD peptide which mimics the typical fibronectin binding sequence arginine-glycine-aspartate had no effect on migration distance. An essential role of the α6β4 heterodimer for migration was ruled out by exposing neurospheres to a blocking integrin α6 antibody, because this treatment had no influence on hNPC migration. That is in contrast to existing data showing that a α6 blocking antibody ,in the same concentration we used (10 µg/ml), exhibit decreased migration in hNPCs compared with cells treated with an isotype-matched antibody control (Flanagan et al. 2006;Ma et al. 2008). Similar results were published in rat forebrain NPCs (Jacques et al. 1998) and mouse gangliomic eminescence NPCs (Tate et al. 2004). The varying outcome in respect to our study could be due to species differences in the two latter cases. In case of the publication of Flanagan et al. the different gestational age of the hNPCs could play a role. It is decribed that other integrin dimers may have overlapping functions with α6 integrins during early cortical development (Schmid and Anton 2003). The hNPCs used in our study was GW16 in contrast to GW23 in the Flanagan study. So it is possible that in our hNPCs another subunit replaced the function of the impaired α6 subunit.

Exposure to a functional blocking integrin β1 antibody reduced the migration distance at concentrations as low as 1 µg/ml and resulted in a disturbed migration pattern at high doses, accompanied by a reduced cell adhesion. Similar to our observations blockage of β1-integrins by antibodies was reported to impede neuronal precursor cell migration on laminin by Jacques et al already 1998, although this was not quantified by morphometric analysis. Many publications concerning the role of β1 integrins in migration of neural precursors *in vitro* and *in vivo* followed (Andressen et al. 2005;Anton et al. 1999;Belvindrah et al. 2007;Dulabon et al. 2000;Forster et al. 2002;Graus-Porta et al. 2001;Kearns et al. 2003;Ma et al. 2008;Tate et al. 2004) showing the importance of this integrin subunit for neural migration. Surprisingly we not only measured a decreased migration distance but we exactly got the same chain-migration phenotype as observed after EGCG treatment. A 50% knockdown of integrin β1 mRNA was not sufficient to block adhesion. Therefrom, it could be assumed that half of the

β1 integrins were sufficient to provide full adhesion. This is further supported by the results of the adhesion assay with the β1 integrin antibody which showed that a very high dose of antibody is necessary to block adhesion. Altogether, the results of the β1 integrin blocking experiments led us to the hypothesis that gallate-containing green tea flavonoids inhibit hNPC migration and adhesion through binding to the laminin matrix thus disrupting the interaction with β1-integrin on the surface of the cells. This hypothesis was confirmed by co-incubation experiments with EGCG and the integrin β1 antibody. Both agents in subliminal doses of 2 μM for EGCG and 2 μg/ml for the antibody resulted in a chain-migratory phenotype with a strong reduction in migration distance. This implies that both agents acted additively in disturbing hNPC migration. The EGCG treatment resulted in a blocked matrix and that effect was further increased by an impaired β1 integrin activity through the antibody. Similar results were reported by Andressen et al. 2005 who showed that on an improper matrix, in their case a fibronectin matrix, the reduction in β1-integrin knockout NSC migration distance is even higher as on a proper laminin matrix.

Previous *in vitro* studies have shown that loss of β1-integrins influences but not completely inhibits migration and morphological differentiation of neurones (Andressen et al. 1998;Rohwedel et al. 1998). Moreover, in chimeric embryos there is a contribution of β1-integrin knockout cells to normal brain development (Fassler and Meyer 1995). But neuronal migration in the developing chick tectum was markedly reduced after retroviral infection with antisense mRNAs of the β1 or α6 integrin subunits (Galileo et al. 1992;Zhang and Galileo 1998) and inhibition of the α3β1 integrin reduced neuronal migration along radial glia *in vitro* (Anton et al. 1999). In an attempt to study the role of β1 integrin in the developing cortex, β1 integrin-floxed mice were crossed with nestin-cre mice, resulting in widespread inactivation of β1 integrins in cortical neurons and glia (Graus-Porta et al. 2001). As outcome cortical layer formation was disrupted in these mice, in large part as a result of defective meningeal basement membrane assembly, marginal-zone formation and glial end feet anchoring at the top of the cortex. Taken together, this evidence supports a role for β1 integrins in regulating neuronal migration during CNS development. A disactivation of the interaction between integrin β1 and the laminin matrix through green tea flavonoids might possibly lead to neurodevelopmentally defects.

At the moment there is to our knowledge no animal study published determining the effects of green tea flavonoids on brain development *in vivo*. Epidemiological studies with women drinking green tea during pregnancy revealed an association between tea consumption and spina befida (Correa et al. 2000) and an increasing risk of a neural-tube defect-affected pregnancies (Li et al. 2006). These effects could be eventually explained by a study by Navarro-Peran et al.. They demonstrated that EGCG is an efficient inhibitor of human dihydrofolate reductase in Caco-2 cells. This results in a disturbed folic acid metabolism in

cells, causing inhibition of RNA and DNA synthesis and altering DNA methylation (Navarro-Peran et al. 2007).

But one should remember that the intake of flavonoids as dietary supplements results in up to 100 times higher plasma concentrations than could be achieved by an usual Western diet (Mennen et al. 2005). In case of EGCG plasma concentrations between 0.3 µM and 7.5 µM were measured after ingestion of one 500 mg capsule (Ullmann et al. 2003). It was shown that flavonoids can enter the fetal brain at least in rats, yielding concentrations of EGCG about one-eighth of that in the maternal plasma (Chu et al. 2006). This means that concentrations about 1 µM are possibly achievable in human fetal brains, which is very close to our obtained EC_{50} value of about 2 µM in the migration assay.

In the past few decades, accumulating data have shown potential beneficial effects of flavonoid-rich foods (rev. in Tapiero et al. 2002) although the contribution of flavonoids to this benefit is uncertain (rev. in Halliwell 2007). This has sometimes led to overestimation of the current knowledge regarding their potential effects. With the disappointing results of the intervention trials with β-carotene supplementation (Anon 1994) in mind, it is to consider that polyphenols may, in specific populations and developmental stages, have effects opposite those that are desired. Based on the entire information of all available data the safety of elevated intakes cannot be assumed and more toxicologic data are urgently needed.

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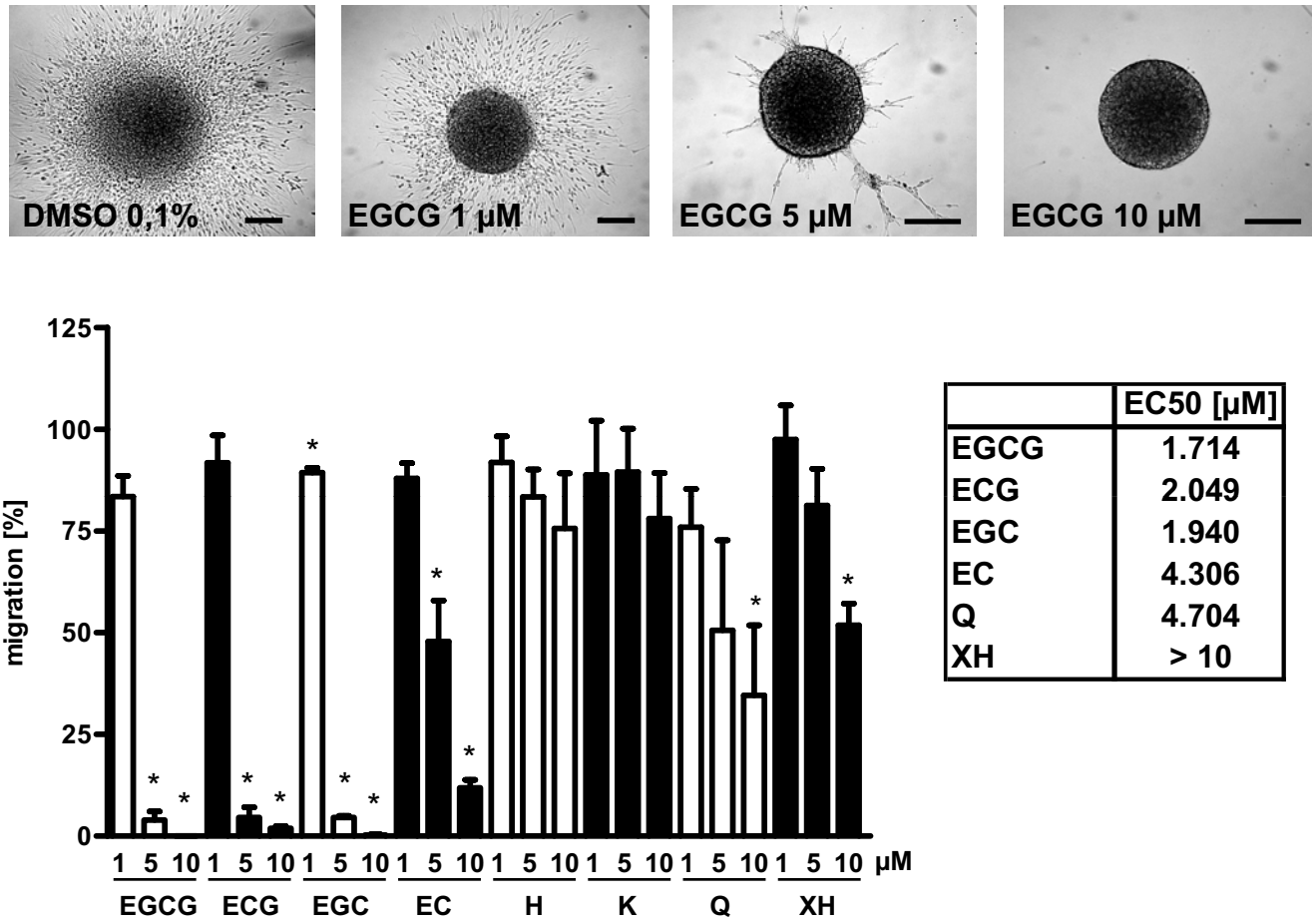


	EC50 [µM]
EGCG	1.714
ECG	2.049
EGC	1.940
EC	4.306
Q	4.704
XH	> 10

Fig. 1: Effects of flavonoids on migration of human neural progenitor cells (hNPCs). Exposure during differentiation for 48h **A.** Microscopic phase-contrast images. Scale bar = 300 µm **B.** Migration distance of human NPCs after exposure with Epigallocatechingallate (EGCG), Epicatechingallat (ECG), Epigallocatechin (EGC), Epicatchin (EC), Hesperetin (H), Kaempherol (K), Quercetin (Q), Xanthohumol (XH). Data represent the mean ± SEM of at least 3 independent experiments (5 spheres/exposure) as % of the solvent control at a laminin concentration of 5 µg/ml. Data were analyzed by Twoway ANOVA (* = $p < 0,05$ vs. solvent control). EC50 values of flavonoids with a significant effect on migration are presented as insert. Similar results were obtained in two additional individuals.

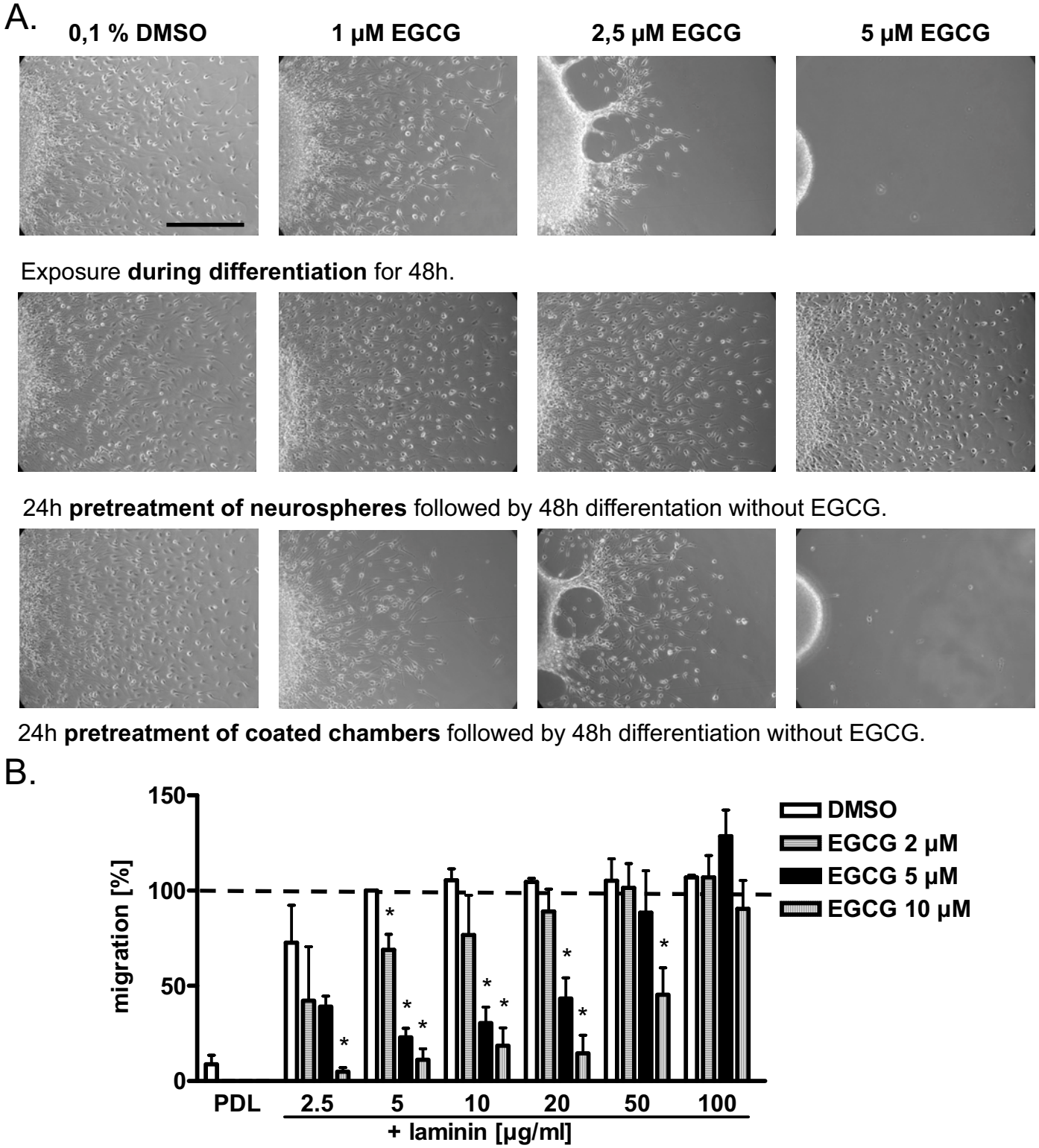
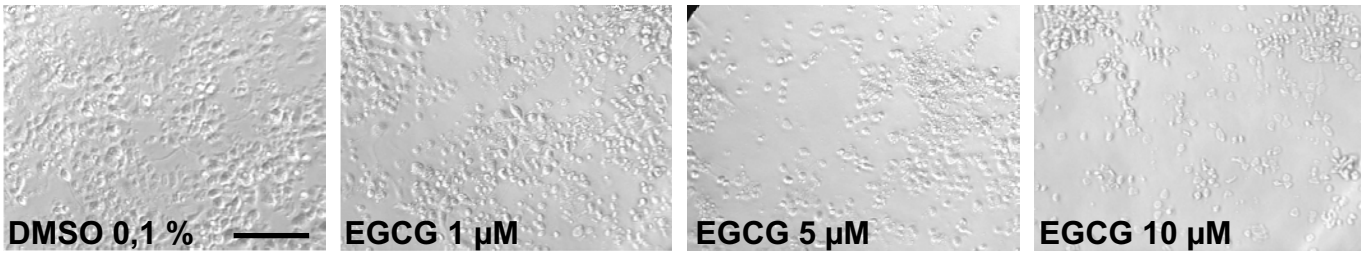


Fig. 2: Flavonoids interact with the extracellular matrix (ECM) and not directly with intracellular signaling. A. Neurospheres were treated as described below the photos. Microscopic phase-contrast images of the EGCG treatment are shown as an example. Similar results were obtained with EGC, EC and Q. Scale bar = 300 µm **B.** The inhibitory effect of EGCG is dependent on the laminin concentration of the coating. The migration distance from the edge of the sphere to the furthest outgrowth was measured. Data represent the mean ± SEM of at least 3 independent experiments (5 spheres/exposure) as % of the solvent control at a laminin concentration of 5 µg/ml. Data were analyzed by Twoway ANOVA (* = $p < 0,05$ vs.control)

A.



B.

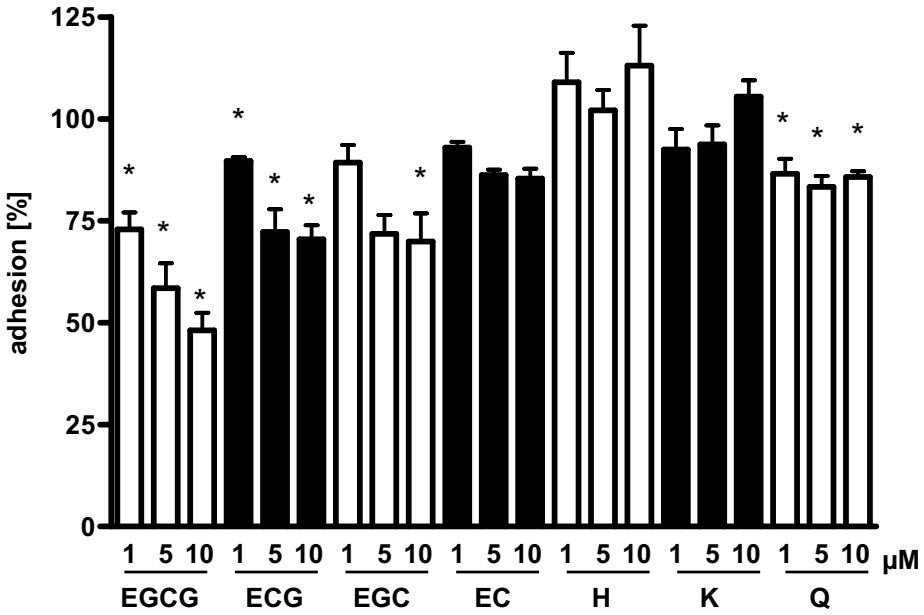


Fig. 3: Flavonoids affect the adhesion of human NPCs. Single cell suspensions of neurospheres were plated onto PDL/laminin coated slides. After 1h exposure with the indicated flavonoids non-adherent cells were removed and the number of remaining cells were measured by CellTiterBlue-Assay **A.** Microscopic phase-contrast images. Scale bar = 100 µm **B.** Number of adherent cells after exposure with different flavonoids. Data represent the mean ± SEM of at least 3 independent experiments (6 spheres/exposure) as % of the solvent control. Data were analyzed by student's t-test with Dunnett post hoc test(* = $p < 0,05$ vs.control) Similar results were obtained an additional individual.

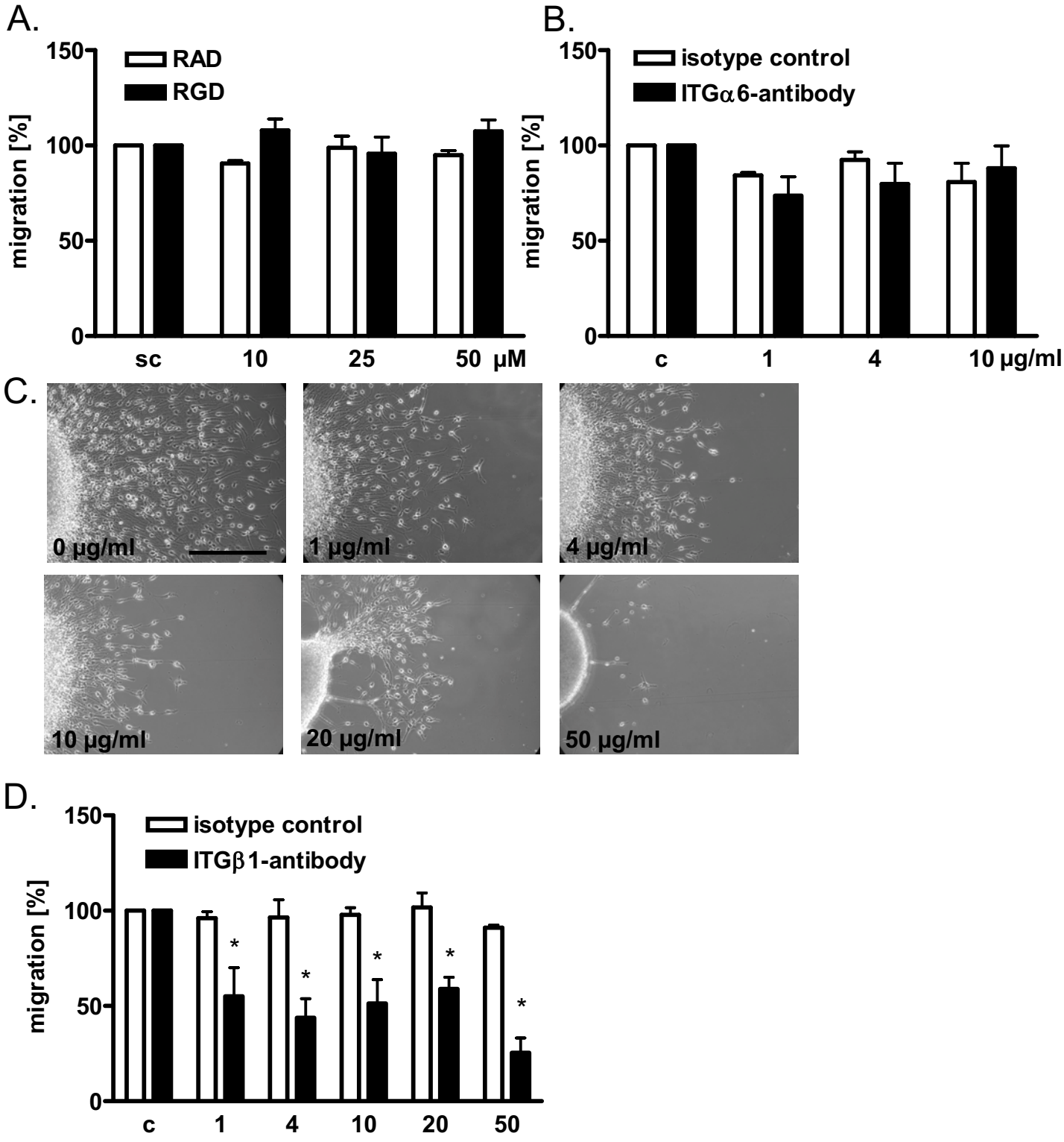


Fig. 4: Effect of a RGD-peptide and specific integrin antibodies on migration of NPCs. Neurospheres were exposed **A.** with RGD (Arg-Glycin-Asp) and the control peptide RAD (Arg-Ala-Asp), with **B.** α6-integrin or **D.** ß1-integrin antibody and the respective isotype control prior differentiation for 1h and during differentiation on PDL/laminin coated slides for further 48h. Data represent the mean ± SEM of at least 3 independent experiments as % of the respective control Data were analyzed by Two-Way ANOVA and Bonferroni post test (* = $p < 0,05$ vs.control). **C.** Microscopic phase-contrast images of β1-integrin treated neurospheres after 48h differentiation. Scale bar = 300 µm

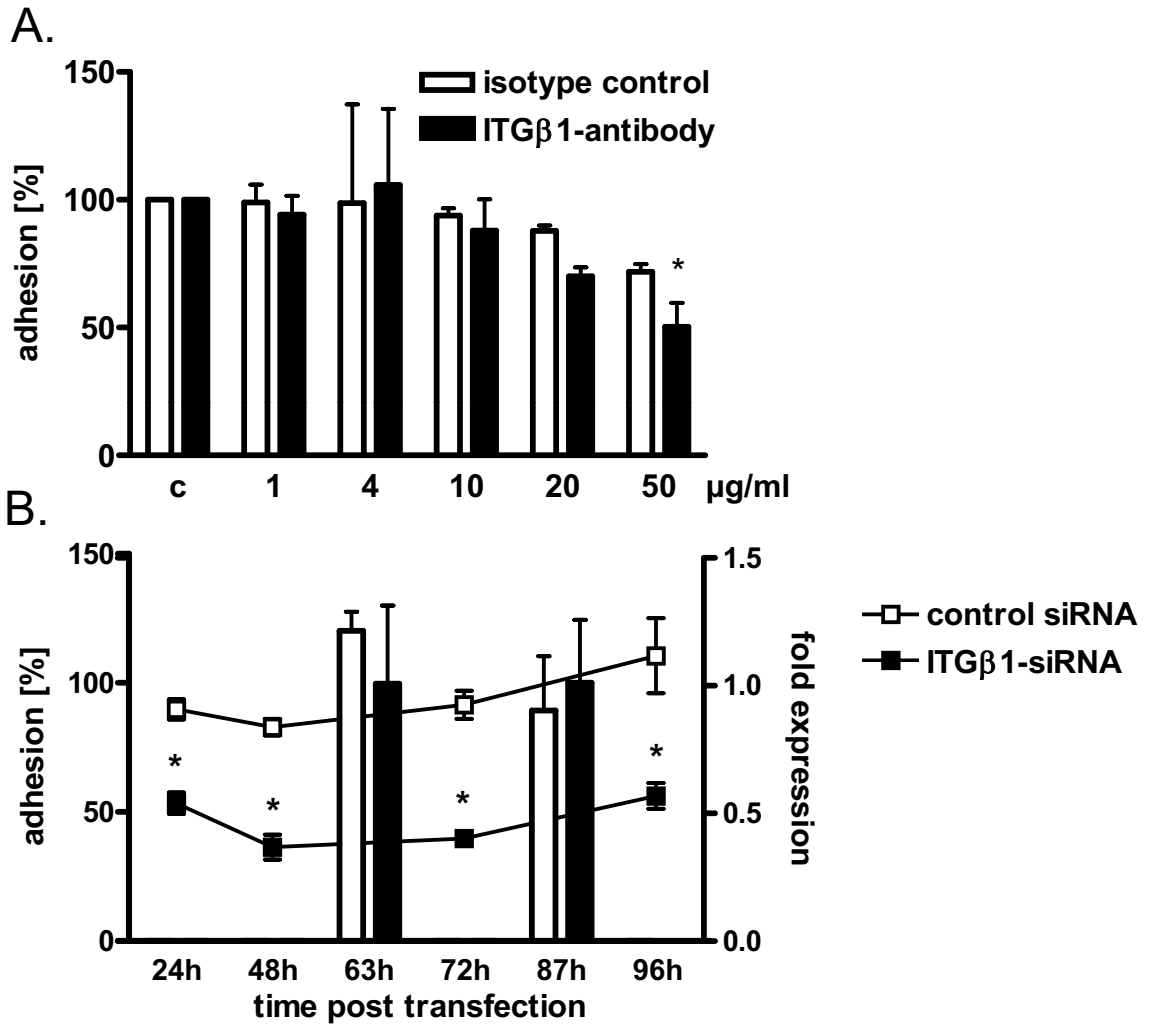


Fig. 5: Blocking of β1-integrin by a specific antibody but not knockdown with siRNA does influence hNPC adhesion. A. Antibody treatment. Dissociated neurospheres were pretreated for 1h with a blocking β1-integrin antibody or isotype-control and then plated onto PDL/laminin coated slides. After 1h non-adherent cells were removed and the number of remaining cells were measured by CellTiterBlue-Assay. Shown are the mean ± SEM of 2-3 independent experiments. Data were analyzed by TwoWay ANOVA with Bonferroni post test (* = $p<0,05$ vs.untreated control). **B.** siRNA knockdown. The percentage of adherent cells 63h and 87h post transfection is shown on the left y-axis as bar diagramm and the relative expression of β1-integrin siRNA after different timepoints post transfection is depicted on the right y-axis as line graph. The siRNA knock down data represent β1-integrin copy numbers normalized to β-actin 24h, 48h, 72h and 96h post transfection. Cells without siRNA treatment served as control and was abitrarily set one. Shown are the mean ± SD of a representative experiment performed in triplicate. The adhesion data describe the percentage of adherent cells as % of the untreated control. Shown are the means ± SD of two independent experiments. Data were analyzed by student's t-test (* = $p<0,05$ vs.untreated control).

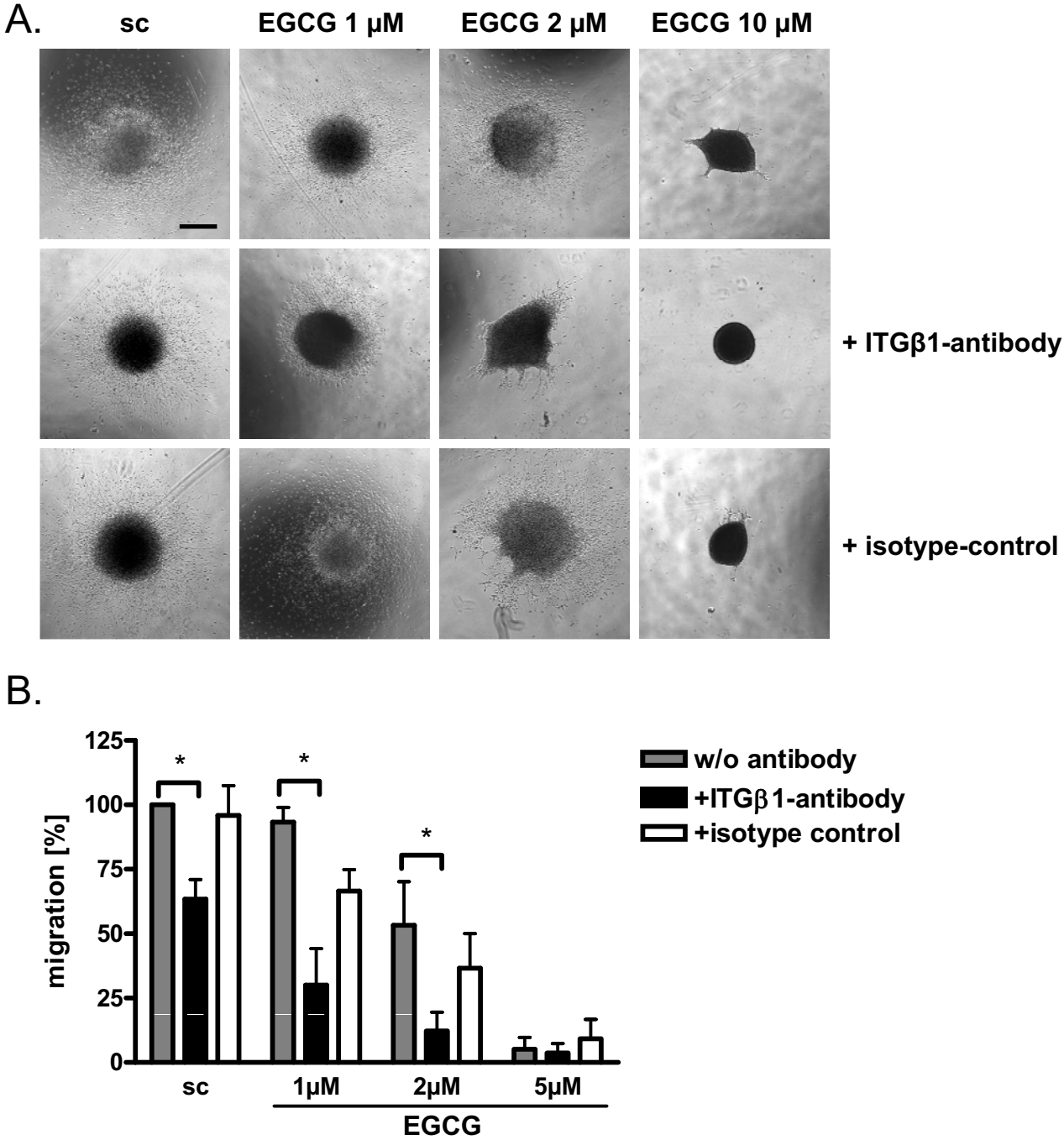
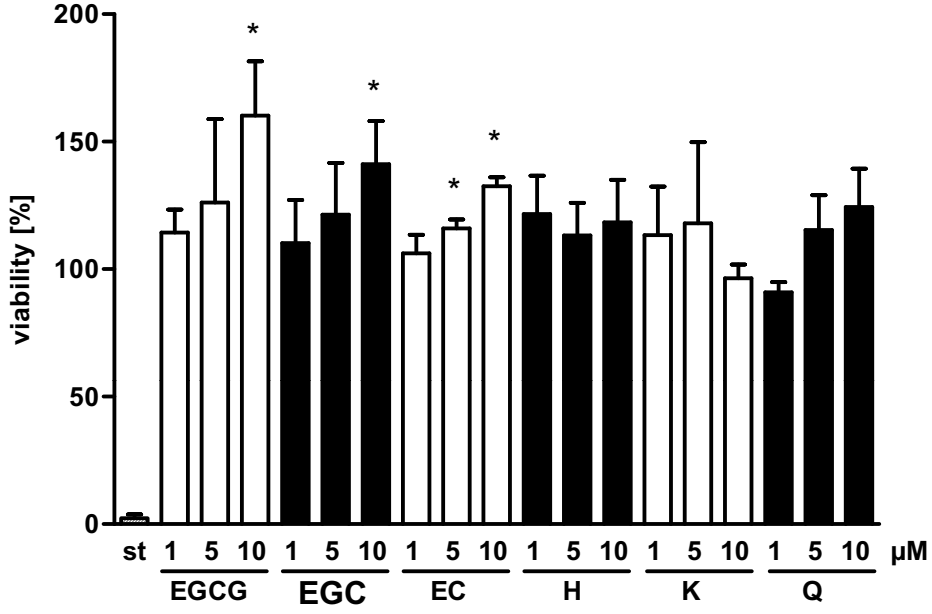


Fig. 5: The combination of β1-integrin antibody and EGCG has an synergistic effect on NPC migration. Neurospheres were exposed with 2 µg/ml ß1-integrin antibody and the respective isotype control prior differentiation for 1h and during differentiation on PDL/laminin coated slides for further 48h with different concentrations of EGCG. **A.** Microscopic phase-contrast images. Scale bar = 500 µm **B.** Migration assay. Data represent the mean ± SEM of 4 independent experiments (5 spheres/exposure) as % of the respective solvent control without antibodies. Data were analyzed by students t-test (* = $p < 0,05$)

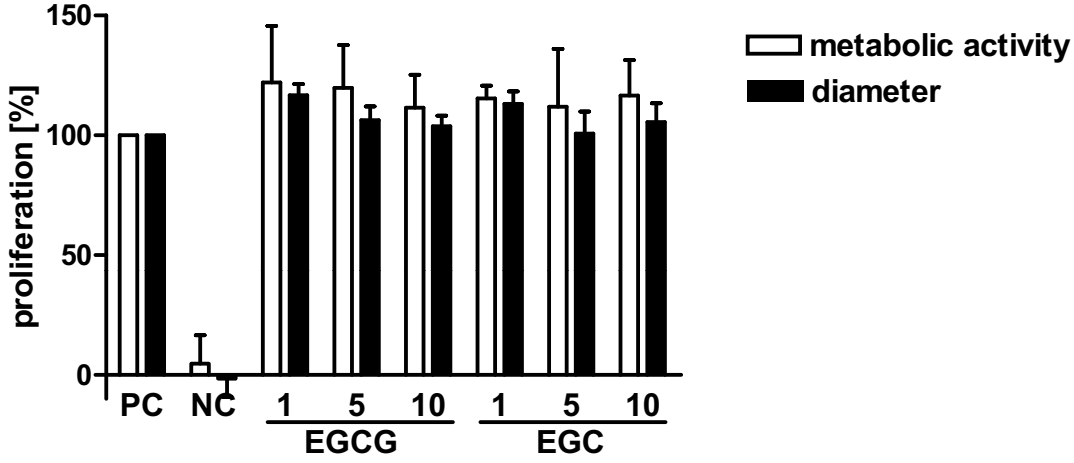
Supplemental Material



n = 3

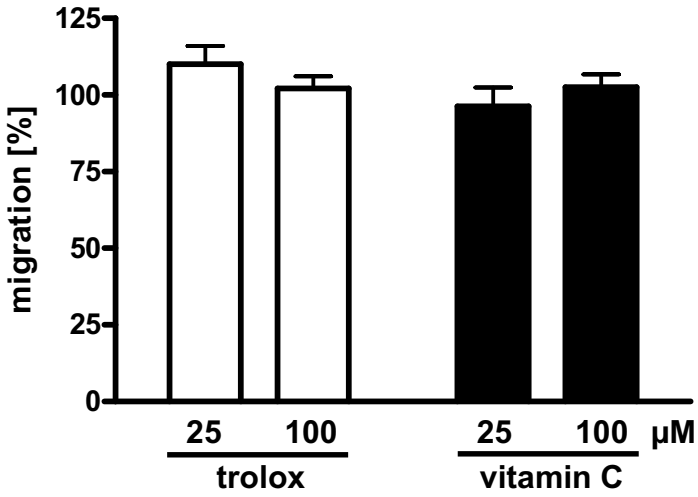
Suppl. Mat. Fig. 1: Flavonoids do not influence NPC viability. Cell viability was measured using CellTiter-Blue Assay and 1 µM staurosporine was chosen as positive control. Data represent the mean ± SEM of 3 independent experiments (3 spheres/exposure) as % of solvent control. Data were analyzed by students t-test (* = $p < 0,05$).

A.



B.

Suppl. Mat. Fig. 2: Flavonoids do not influence NPC proliferation. Proliferation was measured using a combination of CellTiter-Blue Assay which measures metabolic activity and diameter determination. Neurospheres were exposed for 14 days with EGCG and EGC diluted in proliferation medium with (**A.**) or without growth factors (**B.**). Proliferation medium with growth factors serves as positive control (PC) while proliferation medium without EGF and FGF was used as negative control (NC). Data represent the mean ± SEM of at least two independent experiments (6 spheres/exposure) as % of positive control.



Suppl. Mat. Fig. 3: The two antioxidants trolox and vitamin C do not affect NPC migration. Quantification of the migration distance of human NPCs after 48 h exposure with 25 µM and 100 µM trolox and vitamin C. Data represent the mean ± SEM of 3 independent experiments (5 spheres/exposure) as % of solvent control.

3. Abschlussdiskussion

Inhalt der vorliegenden Dissertation war es, die Wirkung verschiedenster AhR-Liganden, die als Verunreinigung in Lebensmitteln oder bewusst als Nahrungsergänzungsmittel aufgenommen werden, auf die humanen neurale Entwicklung *in vitro* zu untersuchen. Dabei war das Ziel, die Rolle des AhR in der humanen neuralen Entwicklung aufzuklären.

3.1 Rolle des AhR in der neuralen Entwicklung

Der Arylhydrocarbon Rezeptor (AhR) ist ein Liganden-aktivierter Transkriptionsfaktor, welcher unter anderem die Expression Fremdstoff-metabolisierender Enzyme und damit den Abbau niedermolekularer chemischer Substanzen reguliert. Darüber hinaus spielt der AhR aber auch eine physiologische Rolle in der Regulation von Zellproliferation und –apoptose (Elferink 2003;Nebert et al. 2000). Für den Menschen gibt es bisher kaum Daten zur physiologischen Rolle des AhR besonders in Bezug auf die neurale Entwicklung. Verschiedene Untersuchungen an Invertebraten, Vertebraten und auch Säugern weisen aber darauf hin, dass sowohl das Fehlen des AhR als auch eine unphysiologische Aktivierung durch exogene Liganden wie TCDD und PAHs entwicklungsneurotoxisch wirken kann (Hays et al. 2002;Henshel et al. 1997;Hill et al. 2003;Kakeyama and Tohyama 2003;Kim et al. 2006;Peterson et al. 1993;Qin and Powell-Coffman 2004;Schantz et al. 1992;Schantz and Bowman 1989).

Die physiologische und entwicklungsspezifische Rolle des AhR im Säuger wurde bisher hauptsächlich anhand unterschiedlicher AhR-defizienter Mäusestämme untersucht. AhR-defiziente Mäuse weisen ein niedrigeres relatives Lebergewicht und eine höhere Apoptoserate der Parenchymzellen auf. Ferner zeigen sich phänotypisch kardiovaskulare Veränderungen, vaskulare Hypertrophie in der Leber und dem Uterus. Zusätzlich weisen sie T-Zell Mangel in der Milz, aber nicht in anderen lymphatischen Organen, Immunsystemdefizienz, und verschiedene dermale Pathologien auf (Esser 2009;Fernandez-Salguero et al. 1997;Gonzalez and Fernandez-Salguero 1998;Lahvis et al. 2000;Schmidt et al. 1996). Über Veränderungen in der neuralen Entwicklung oder neurologische Störungen durch ein Fehlen des AhR ist nichts berichtet, wobei man erwähnen muss, dass solche Untersuchungen nicht im Fokus der Studien standen oder auch möglicherweise bisher nicht durchgeführt wurden.

Im Gegensatz dazu scheint eine unphysiologische Aktivierung des AhR durch Dioxine oder PAHs einen Einfluss auf Lernen und Gedächnis bei Nagern und auch bei Affen zu haben (Markowski et al. 2001;Schantz et al. 1996;Schantz and Bowman 1989;Seo et al. 1999;Seo et al. 2000). Dabei waren die Testergebnisse besonders bei niedrigen TCDD Dosen äußerst widersprüchlich und reichten von deutlicher Verbesserung der Lern- und Gedächnisleistung (Schantz et al. 1996;Seo et al. 1999) bis zu reduzierter kognitiver Leistungsfähigkeit (Schantz and Bowman 1989;Seo et al. 2000). Ob die verbesserte Lern- und Gedächnisleistung bei niedrigem Expositionslevel ein Anzeichen für kompensatorische Veränderungen auf eine toxische Wirkung des TCDDs sind, konnte bisher noch nicht geklärt werden. Es ist jedoch beobachtet worden, dass TCDD die Zusammensetzung des für die Gedächnisleistung wichtigen NMDA-Rezeptors im Gehirn von Ratten verändert, indem es die mRNS Expression der NR2B Untereinheit inhibiert und gleichzeitig die Expression der NR2A Untereinheit stimuliert (Kakeyama et al. 2001). Darüber hinaus führt die Aktivierung des AhR zu akuten physiologischen Veränderungen in neuralen Geweben. Mehrere Studien in Nagermodellen konnten zeigen, dass AhR-Agonisten oder eine Überexpression des AhR die intrazelluläre Calciumhomöostase sowohl in Neuronen als auch Gliazellen beeinflussen (Hanneman et al. 1996;Hong et al. 1998;Legare et al. 1997), die Differenzierung von Gliom- und Neuroblastomzellen modellieren (Akahoshi et al. 2006;Takanaga et al. 2004), die Neurogenese und Viabilität von cerebralen granulären Neuroblasten beeinflussen (Kim and Yang 2005;Williamson et al. 2005) und die Zell-Zell-Kommunikation in primären Hippocampus-Neuronen stören (Legare et al. 2000). Dies sind erste Anhaltspunkte, die Hinweise auf mögliche molekulare Mechanismen geben. Dabei kann jedoch nicht ausgeschlossen werden, dass einige Effekte auf AhR-unabhängige Mechanismen zurückzuführen sind. Dies wurde bereits sind für viele andere vor allem TCDD-vermittelte Wirkungen beschrieben (Akintobi et al. 2007;Dohr et al. 1997;Kerkvliet et al. 1990;Shipley and Waxman 2006).

Im Menschen stellt sich die Situation wie folgt dar. Es gibt eine Reihe von Studien, die entwicklungsneurotoxische Störungen bei Kindern von rauchenden Müttern oder Müttern, die während der Schwangerschaft PCB-, Dioxin- oder PAH-kontaminierte Nahrung aufgenommen haben, beschreiben (Batty et al. 2006;Fried et al. 1998;Grandjean and Landrigan 2006;Kallen 2000;Lindley et al. 1999;Olds et al. 1994;Wormley et al. 2004). Ähnlich wie bei den Studien an Nagern ist aber auch hierbei unklar inwieweit der AhR bei den beobachteten Effekten auf kognitive Fähigkeiten und Verhalten eine Rolle spielt. Darüber hinaus handelt es sich bei Zigarettenrauch und kontaminierter Nahrung fast immer um eine Mischexposition mit einer ganzen Reihe von toxischen Substanzen, d.h. es kann nur schwer definiert werden, wie groß der Anteil der einzelnen Komponenten an den beobachteten Effekten ist.

Die im Rahmen der vorliegenden Promotion generierten Daten zeigen noch einen weiteren zu bedenkenden Faktor in der Bewertung der Rolle des AhR in der neuralen Entwicklung auf. Durch die Messung verschiedener Endpunkte nach Belastung mit AhR-Agonisten und eines AhR-Antagonisten parallel in humanen und murinen neuralen Progenitorzellen (NPCs) konnte gezeigt werden, dass es große Speziesunterschiede in der Suszeptibilität für AhR-vermittelte ENT gibt. Die AhR-Agonisten 3-MC und B(a)p führen zu einer verringerten Migration und der AhR-Antagonist MNF zu einer inhibierten Proliferation in murinen NPCs wohingegen alle 3 Substanzen keinen Effekt im humanen Modell haben. mRNS-Expressionsanalysen zeigten, dass die Ursache für die Unterschiede in einer deutlich niedrigeren Expression der AhR-Signalwegkomponenten in humanen NPCs liegt. Dies hat zur Folge, dass es durch gleiche Konzentrationen 3-MC in humanen Neurosphären wahrscheinlich im Gegensatz zu murinen Neurosphären zu keiner Aktivierung des AhR kommt. Klassische AhR-Zielgene wie CYP1A1 und CYP1B1 werden in humanen NPCs nicht durch 3-MC in ihrer Expression beeinflusst und es ist davon auszugehen, dass dieses Ergebnis auf die gesamte AhR-regulierte Genbatterie übertragen werden kann.

Der AhR ist in seiner Struktur evolutionär stark konserviert. Gleichzeitig ist bekannt, dass es teilweise große Unterschiede in der Toxizität von AhR-Liganden in verschiedenen Spezies gibt. So liegt z. B. für Nagetiere die LD_{50} für 2,3,7,8-TCDD beim Meerschweinchen bei 0,6-2,1 µg/kg, bei der Ratte bei 22-60, bei der Maus zwischen 114 und 184, beim Goldhamster bei 1147-5051 µg/kg KG (Hengstler et al. 1999;Kociba and Schwetz 1982). Das von uns beschriebene spezies-spezifische Expressionslevel wird durch Untersuchungen zur Schließung der Gaumenspalte nach TCDD Exposition im Vergleich zwischen einem humanen und einem murinen Modell unterstützt (Abbott et al. 1999). Abbott et al konnte zeigen, dass in ihrem humanen *in vitro* System bis zu 200-fach höhere TCDD-konzentrationen nötig sind, um ähnliche entwicklungstoxische Effekte wie im murinen *in vitro* System hervorzurufen und das dies mit einem deutlich verringerten Expressionslevel der AhR-Signalwegkomponenten verknüpft ist (Abbott et al. 1989;Abbott et al. 1998;Abbott et al. 1999). Eine Ursache für die spezies-spezifische Suszeptibilität gegenüber AhR-vermittelter Toxizität liegt in der unterschiedlichen Affinität des AhR für seine Liganden in verschiedenen Spezies (Ema et al. 1994;Flaveny et al. 2009;Harper et al. 1988;Moriguchi et al. 2003;Ramadoss and Perdew 2004). Diese Möglichkeit wurde innerhalb der vorliegenden Studie nicht näher untersucht, könnte aber durchaus zu den Ergebnissen beigetragen haben.

Zusammenfassend kann gesagt werden, dass die Exposition mit klassischen AhR-Liganden zu Störungen in der neuralen Entwicklung bei Labornagern führt. Ob ein Fehlen oder die permanente Inaktivierung des AhR in Nagern ebenfalls wie in Invertebraten ENT zur Folge hat, können nur weiterführenden Studien mit entsprechenden AhR knock-out Tieren klären.

Beim Menschen gibt es das große Problem der permanenten Mischexposition mit unterschiedlichen Toxinen, so dass sich eine Abhängigkeit vom AhR schwer nachweisen lässt. Die Daten der vorliegenden Dissertation und Befunde aus verschiedensten anderen Studien weisen jedoch darauf hin, dass der Mensch deutlich unempfindlicher in Bezug auf AhR-vermittelte Wirkungen zu sein scheint (Connor and Aylward 2006;Okey et al. 1994;Silkworth et al. 2005). Dieser Punkt hat besondere Wichtigkeit in der Regulation der entsprechenden Substanzen, da die Grenzwertsetzung bisher nach wie vor auf der Extrapolation von Labornagerdaten beruht.

3.2 PBDE und Flavonoide stören die humane neurale Entwicklung *in vitro* aufgrund alternativer Mechanismen

In der vorliegenden Dissertation konnte gezeigt werden, dass sowohl PBDE als auch Flavonoide einen Einfluss auf verschiedene Endpunkte der humanen neuralen Progenitorzellentwicklung haben. PBDE und Flavonoide führen beide zu einer verringerten Migration und PBDE beeinflussen darüber hinaus auch die Differenzierung der Zellen. Beide Substanzklassen haben in den getesteten Konzentrationen keinen Einfluss auf die Zellviabilität und –proliferation. Durch die zuvor beschriebenen Ergebnisse zur Rolle des AhR in der humanen neuralen Gehirnentwicklung *in vitro* kann geschlossen werden, dass die nach PBDE und Flavonoidexposition beobachteten Veränderungen nicht auf eine Aktivierung oder Inhibierung des AhR zurückgeführt werden können. In der aktuellen Literatur gibt es sogar Hinweise darauf, dass die in dieser Studie verwendeten Flavonoide nur in sehr hohen Konzentrationen und PBDE, wenn überhaupt, nur im geringen Maße mit dem AhR wechselwirken können. Diese Befunde werden im Folgenden kurz zusammengestellt.

Bisherige Studien über Interaktionen von PBDE mit dem AhR fokussierten hauptsächlich auf die Expression und Aktivität des CYP1A1 als Biomarker des AhR-Signalwegs. Einige Berichte in der Literatur belegen eine Induktion der CYP1A1-Expression durch PBDE (Behnisch et al. 2003;Chen and Bunce 2003;Zhou et al. 2002), andere wiederum zeigten keine Induktion und zudem gibt es Studien, welche sogar eine inhibitorische Wirkung der PBDE auf die CYP1A1-Expression demonstrierten (Chen and Bunce 2003;Kuiper et al. 2004;Peters et al. 2006). Wahl et al. wies 2008 in verschiedenen Modellsystemen nach, dass die Ursache der widersprüchlichen experimentellen Befunde in Verunreinigungen mit den als AhR-Agonisten beschriebenen bromierten Furanen in kommerziellen PBDE-Mixturen liegt und konnte damit eine Beteiligung des AhR an den beobachteten Effekten ausschließen. Aus diesem Grund kann nach momentanem Stand der Literatur keine mechanistische

Verbindung zwischen AhR-Aktivität, CYP1A1-Induktion und PBDE-vermittelter Toxizität hergestellt werden.

Wie eingangs beschrieben fungieren Flavonoide als Agonisten und/oder Antagonisten des AhR und der AhR-vermittelten Genexpression insbesondere der CYP-Induktion. Einige Verbindungen wie Quercetin, Galangin und Tangeretin haben sowohl agonistisches als auch antagonistisches Potential. Bisherige Veröffentlichungen über die in dieser Dissertation im Vordergrund stehenden Grünteeflavonoide weisen darauf hin, dass diese eine AhR-/CYP-Aktivierung durch TCDD oder PAHs inhibieren, aber gleichzeitig als einzeln verabreichte Substanzen zur Aktivierung des AhR und/oder CYP-Enzymen führen können (Ashida et al. 2000;Liu et al. 2003;Moon et al. 2006;Netsch et al. 2006;Palermo et al. 2003). Diesen Arbeiten ist gemein, dass die entsprechenden Grünteeflavonoide erst in Konzentrationen von 50-200 µM wirksam waren. Diese liegen deutlich oberhalb der in dieser Arbeit verwendeten und in biologischen Geweben gemessenen Konzentrationen, so dass davon auszugehen ist, dass selbst bei einer ausreichenden Expression der AhR-Signalwegkomponenten eine AhR-Aktivierung/-Inaktivierung kein Faktor für die dargestellten Effekte nach Grünteeflavonoidexposition sein kann.

Dies führt zu der Schlussfolgerung, dass PBDE und Flavonoide höchstwahrscheinlich ihre Wirkungen auf humanen neurale Progenitorzellen über alternative Mechanismen ausüben. Die Publikationen 2.4-2.6 zeigen, dass an der ENT dieser Substanzen endokrine Disruption der TH-Signaltransduktion, Störungen der Calcium-Homöostase sowie der Integrin-EZM-Interaktion beteiligt sind. Die aus diesen Arbeiten hervorgegangenen Befunde werden in den nächsten Abschnitten diskutiert.

3.2.1 Endokrine Disruption

PBDE-Kongenere zeigten in Tierversuchen entwicklungsneurotoxisches Potential (Costa and Giordano 2007). So konnten in Experimenten mit Ratten, die während der neonatalen Gehirnentwicklung PBDE-Kongeneren ausgesetzt waren, neurologische Defekte im adulten Tier festgestellt werden (Eriksson et al. 2001;Eriksson et al. 2002). Nach BDE99-Exposition kam es zu einer Beeinträchtigung der spontanen motorischen Aktivität, einer Veränderung der cholinergen Transmitter-Suszeptibilität und einer Disruption der Habituations-Fähigkeit (Viberg et al. 2002). Die PBDE-Kongenere BDE47, BDE99, BDE153 und BDE209 führten in Mäusen und Ratten generell zu einer Beeinträchtigung des Lernens und des Gedächtnisses (Eriksson et al. 2001;Eriksson et al. 2002;Eriksson et al. 2006;Viberg et al. 2003a;Viberg et al. 2003b;Viberg et al. 2004;Viberg et al. 2006;Viberg et al. 2007;Viberg et al. 2008). Die molekularen Mechanismen der toxischen Wirkung von PBDE Kongeneren auf das kognitive

System und das Verhalten sind unklar. Auf zellulärer Ebene wurden *in vitro* akute und subakute PBDE-Effekte berichtet, z.B. Interferenzen von PBDE-Kongeneren mit intrazellulären Signalkaskaden im Gehirn (Kodavanti and Ward 2005) sowie die Freisetzung von 3H-Arachidonsäure durch DE-71 in cerebralen Granularzellen von Ratten (Kodavanti and Derr-Yellin 2002). Weitere toxische Effekte, welche im Zusammenhang mit der Gehirnentwicklung stehen könnten, sind Wirkungen auf das Thyroidhormonsystem.

Während der normalen Gehirnentwicklung sind Schilddrüsenhormone essentiell für die Steuerung von neuraler Migration und die Reifung von Neuronen und Gliazellen (Alvarez-Dolado et al. 1999;Wong and Leung 2001). Daher führt eine Schilddrüsenhormonunterversorgung während der embryonalen und fetalen Entwicklung zu einer großen Anzahl von neuroanatomischen Defekten und Verhaltensauffälligkeiten (Haddow et al. 1999;Schalock et al. 1977;Zoeller and Crofton 2005). Da ähnliche Verhaltensveränderungen auch bei PBDE-exponierten Nagern auftraten und in mehreren Studien nach PBDE-Belastung während der Trächtigkeit ein verringerter Schilddrüsenhormonspiegel in den Muttertieren und/oder Nachkommen festgestellt worden war (Costa et al. 2008), lag der Schluss nahe, dass PBDE endokrine Disruptoren des Schilddrüsenhormonsystems sein könnten. Als zugrunde liegender Mechanismus dieser Disruption wurden folgende zwei Szenarien diskutiert: zum einen eine gesteigerte T4-Exkretion durch Induktion von an der Biotransformation beteiligten Enzyme und zum anderen die Kompetition von PBDE-Metaboliten mit T4 um die Bindung an das Thyroidhormon-Transport-Protein Transthyretin (Hallgren et al. 2001;Meerts et al. 2000;Zhou et al. 2001). Auch die den PBDE strukturverwandten PCB wirken als endokrine Disruptoren des TH-Systems. Neben systemischen Effekten wurden auch direkte Interferenzen mit der zellulären TH-Signaltransduktion *in vitro* (Fritsche et al. 2005) und *in vivo* (Zoeller and Crofton 2000) beobachtet. Wir konnten nun mit unseren Arbeiten zeigen, dass PBDE auch in die zelluläre TH-Homöostase eingreifen können und somit zur Verzögerung der Migration und neuralen Differenzierung führen. Interessanterweise haben PBDE einen gegenläufigen Effekt auf die Oligodendrozytendifferenzierung im Vergleich zu PCB. Während PCB118 die Oligodendrozytendifferenzierung akzeleriert, wird diese durch PBDE inhibiert.

Aus diesen Ergebnissen kann gefolgert werden, dass PBDE nicht nur systemisch, sondern auch auf zellulärer Ebene als endokrine Disruptoren agieren. Die Daten unserer Studie deuten darüber hinaus darauf hin, dass PBDE direkt mit dem Thyroidhormonrezeptorkomplex wechselwirken können und ihn so in seiner Aktivität inhibieren. Somit haben wir erstmalig einen dritten Mechanismus der endokrinen Disruption des TH-Signalweges beschrieben. Unsere Hypothese wird bisher durch die Ergebnisse einer *in vivo* Studie mit Dickkopfelritzen gestützt, in der demonstriert wurde, dass es nach BDE-47

Belastung spezifisch zu Veränderungen in der Expression von Thyroidhormonrezeptor-regulierten Genen kommt (Lema et al. 2008).

3.2.2 Störung der intrazellulären Calciumhomöostase

Ca^{2+}-Ionen dienen als sekundäre Botenstoffe und die freie intrazelluläre Calciumkonzentration, $[Ca^{2+}]_i$, nimmt bei einer Vielzahl neuronaler Funktionen, wie etwa bei der synaptischen Übertragung, bei der Kontrolle der neuronalen Erregbarkeit oder bei neuronalen Lernvorgängen, aber auch für das neuronale Überleben eine zentrale Stellung ein (Berridge 1998;Gnegy 2000). Aktuelle Forschungsergebnisse zeigen, dass Veränderungen in der Calciumhomöostase und -signalauslösung zu verschiedensten Pathologien führen können, die nicht nur auf das sich entwickelnde Gehirn beschränkt sind, sondern auch neurodegenerative Krankheitsbilder wie Alzheimer, Epilepsie, Ischämie und Autismus mit einschließen (Castaldo et al. 2009;Gargus 2009;Kawahara et al. 2009;Wojda et al. 2008). Für die Realisierung sowohl zeitlich als auch örtlich hoch aufgelöster Ca^{2+} Signale können Calciumionen aus dem extrazellulären Raum, über Kanäle in der Plasmamembran in die Zelle gelangen, aber auch aus intrazellulären Speichern freigesetzt werden. Die intrazelluläre Calciumkonzentration liegt unter physiologischen Bedingungen 1000-fach unterhalb der extrazellulären Konzentration. Für die Aufrechterhaltung dieses Calciumgradienten zwischen dem Extra- und dem Intrazellularraum sind zwei Proteine essentiell: eine ATP-abhängige Calciumpumpe und ein Na^+/Ca^{2+}-Austauscher (Augustine and Neher 1992;Carafoli 1991;Neher and Augustine 1992). Darüber hinaus kann die Zelle Ca^{2+} auch intrazellulär im endoplasmatische Retikulum (ER), den Mitochondrien und im Nucleus speichern (Gerasimenko and Gerasimenko 2004;Reyes and Parpura 2009). Ein intrazelluläres Calciumsignal kann entweder über die Öffnung Calcium-permeabler Ionenkanäle in der Zellmembran oder Ca^{2+} Freisetzung aus dem ER und den Mitochondrien ausgelöst werden. Dabei handelt es sich bei den Ionenkanälen in der Plasmamembran entweder um spannungsgesteuerte oder um ligandenkontrollierte Kanäle. Aus intrazelluären Speichern kann Ca^{2+} über die Aktivierung des Inositol-1,4,5-triphosphat-Rezeptors (IP_3R) und/oder des Ryanodin-Rezeptors (RYR) freigesetzt werden (Ehrlich and Bezprozvanny 1994). Schließlich verfügen Nervenzellen über Calcium-bindende Proteine, z.B. Calbindin, die in der Lage sind als Calcium-Puffer zu dienen und damit zusätzlich die Größe und die Kinetik des Calciumsignals zu modifizieren (Heizmann 1992;Heizmann and Hunziker 1991).
Ziel der vorliegenden Untersuchungen war, herauszufinden, ob PBDE die Calciumhomöostase humaner neuraler Progenitorzellen stören. Dabei wurde untersucht, ob (i) eine Langzeitbelastung mit PBDEs zu einer Modulation z.B. von Kanal- oder

Rezeptorexpressionen führt, die sich in einer veränderten Reaktion auf extrazelluläre Stimuli äußert, und ob (ii) PBDE akute Effekte auf die Calciumhomöostase haben. Unsere Ergebnisse zeigen, dass eine Langzeitexposition mit 10 µM BDE-47 und BDE-99 über sieben Tage keinen Einfluss auf die Anzahl der reagierenden Zellen und die Amplitude des Calciumsignals hat. Im Gegensatz dazu führt eine akute Belastung mit BDE-47 (2 µM) und seinem hydroxylierten Metaboliten 6-OH-BDE (0,2 µM) zu einem signifikanten Anstieg der intrazellulären Ca^{2+} Konzentration. Weiterführende Experimente mit verschiedenen Blockierungsreagenzien führten zu dem Ergebnis, dass der Anstieg des intrazellulären Calciumspiegels in den NPCs nach PBDE-Belastung sowohl durch Einstrom aus dem umgebenen extrazellulären Medium als auch durch Entleerung intrazellulärer Speicher wie des endoplasmatischen Retikulums und der Mitochondrien erfolgt. Mit diesen Daten konnten wir die Ergebnisse einer Studie mit PC-12 Zellen bestätigen (Dingemans et al. 2008). Die Aufklärung des zugrunde liegenden Mechanismus ist Gegenstand weiterführender Forschungen. Es konnte bisher lediglich eine Aktivierung des RYR, die für die strukturverwandten PCB beschrieben ist (Gafni et al. 2004;Ta and Pessah 2007), ausgeschlossen werden, da in unserem Modellsystem der RYR nicht funktional exprimiert zu sein scheint.

Besonders bedenklich ist, dass der BDE-47 Metabolit 6-OH-BDE eine deutlich stärkere Potenz zeigt, ein Calciumsignal auszulösen. Die Entstehung von hydroxylierten PBDE-Metaboliten *in vitro* und *in vivo* ist vielfach in der Literatur beschrieben (Hakk and Letcher 2003). Experimentelle Studien von Meerts und Mitarbeitern (2000; 2001) belegen die Entstehung von solchen hydroxylierten PBDE-Metaboliten nach Inkubation der PBDE-Kongenere in induzierten Leber-Mikrosomen und die Existenz hydroxylierter PBDE-Metabolite konnte auch *in vivo* in Fäzes und Urin von Ratten und Mäusen nachgewiesen werden, wobei der Anteil an hydroxlierten Metabolite bis zu 85% betrug und nur ein geringer Teil unmetabolisiert ausgeschieden wurde (de Wit 2002;Hakk and Letcher 2003).

3.2.3 Beeinflussung der Zell-EZM Interaktion

Für das sich entwickelnde humane Nervensystem ist die Interaktion zwischen neuralen Progenitorzellen und extrazellulärer Matrix essentiell. Zwei aktuelle *in vitro* Studien mit humanen NPC konnten zeigen, dass besonders die Interaktion zwischen dem extrazellulären Matrixprotein Laminin und β1-Integrinen auf der Zelloberfläche wichtige Funktionen wie Proliferation, Migration und Differenzierung steuert (Flanagan et al. 2006;Ma et al. 2008). Auch eine Reihe von *in vivo* Studien an Nagern und Vögeln, bei denen entweder Integrine und Laminine akut blockiert oder komplett ausgeknockt wurden, demonstrieren die Notwendigkeit dieser Interaktion für eine normale neurale Entwicklung (Breau et al.

2006;Bronner-Fraser 1986;Georges-Labouesse et al. 1998;Goh et al. 1997;Graus-Porta et al. 2001;Pietri et al. 2004;Tucker 2004).

In dieser Promotion konnte nun gezeigt werden, dass als Nahrungsergänzungsmittel frei verkäufliche Grünteeflavonoide die Migration von humanen NPC inhibieren. Die beiden Gallat-enthaltenen Grünteeflavonoide (EGCG & ECG) stören die Migration über eine Verringerung der Adhäsion an die EZM, während EGC und EC die Migration wahrscheinlich nicht über die Störung der Adhäsion hemmen. Um ausschließen zu können, dass eine individuelle Suszeptibilität der inhibierenden Wirkung der Flavonoide des Grünen Tees zugrunde liegt, wurden die Endpunkte Migration und Adhäsion in einem zweiten Individuum reproduziert. Dabei stammen die Zellen des ersten Individuums aus der 16. Gestationswoche eines männlichen Fetus und die hNCPs des zweiten Individuums wurden aus einem weiblichen Fetus der 17. Gestationswoche isoliert (Zschauer Diplomarbeit 2008).

Die Daten sind im Einklang mit einer Reihe von Studien, die sich überwiegend mit dem Migrations- und Invasionsverhalten von Krebszelllinien beschäftigen, in denen auch häufig ein Unterschied in der Wirkungsstärke zwischen Flavonoiden mit und ohne Gallatgruppe festgestellt werden konnte (Benelli et al. 2002;Bracke et al. 1987;Hung et al. 2005;Liu et al. 2001;Lo et al. 2007;Suzuki and Isemura 2001). Die Wirkung von EGCG auf normale neurale Zellen ist demgegenüber noch kaum untersucht. Einzig für Neurosphären der Ratte ist eine Inhibierung der Migration und Adhäsion durch EGCG bereits beschrieben (Chen et al. 2003). Diese Studie traf aber keine Aussage zu den zu Grunde liegenden Mechanismen. Unsere mechanistischen Untersuchungen lassen den Rückschluss zu, dass eine Störung der Interaktion zwischen der EZM und dem β1-Integrin durch Bindung von EGCG und ECG an Laminin den beschriebenen Effekten zu Grunde liegt.

Diese Hypothese wird durch aktuelle Literaturdaten unterstützt. Eine Inhibierung der Adhäsion durch EGCG und ECG an die extrazellulären Matrixproteine Kollagen und Laminin wurde schon bei glatten Muskelzellen der Ratte beobachtet, als mögliche Interaktion wurde eine Wechselwirkung mit der Integrin β1-Untereinheit genannt. In dieser Studie wurde zudem die Bindungsaffinität des EGCG an das Laminin bestimmt (Lo et al. 2007). Eine ähnliche Studie mit Maus Melanom B16 Zellen konnte ebenso eine Inhibierung der Adhäsion dieser Zellen an Laminin durch Interaktion des EGCG mit Laminin zeigen, sowie eine Bindungsaffinität des EGCG an das Protein Laminin über eine Affinitätschromatographie nachweisen (Suzuki and Isemura 2001).

Die Idee, dass Substanzen über die Blockierung der Zelladhäsion entwicklungsneurotoxisch sind, ist nicht neu. Auch für andere bekannte entwicklungsneurotoxische Substanzen wie Quecksilber, Cadmium, Blei und Ethanol sind Wirkungen auf die Zelladhäsion als zugrunde liegender Mechanismus in der Diskussion (Bearer 2001;Garza et al. 2006;Prozialeck et al. 2002), wobei dabei aber eher Cadherine und Zelloberflächenproteine der Immunglobulin-

Superfamilie wie z.B. das N-CAM und das L1-CAM im Vordergrund stehen. So gibt es beispielsweise eine Mutation des L1-Gens beim Menschen, welche zu neuropathologischen Veränderungen führt. Da diese Hirnentwicklungsstörungen den morphologischen Veränderungen nach pränataler Ethanolexposition ähneln, wurde die Hypothese gerneriert, dass die Toxizität von Ethanol durch die Wirkung auf das L1 verursacht werden könnte (Bearer 2001;Goodlett et al. 2005).

Demgegenüber wirken Schwermetalle wie Blei, Quecksilber und Cadmium hauptsächlich über die Modulation der Cadherin-abhängigen Zelladhäsion. Cadherine sind Calcium-abhängige Zelladhäsionsmoleküle, die Zell-Zell-Interaktionen ermöglichen. Funktionsverlust durch Blockierung durch Antikörper oder Veränderungen auf Genebene führen auch in diesem Fall zu Störungen während der neuralen Entwicklung (Bronner-Fraser et al. 1992;Chazal et al. 2000;Detrick et al. 1990;Grunwald 1993;Lagunowich et al. 1994;Tepass et al. 2000). Es werden drei verschiedene Mechanismen der Schwermetalltoxizität im Bezug auf Cadherine diskutiert: (i) Schwermetalle verdrängen Calcium von den Bindungsstellen (ii) Modulation der Expression und Prozessierung von Cadherinen (iii) Beeinflussung der Cadherin-Catenin-Bindung (Prozialeck et al. 2002). Somit ist die Störung der Hirnentwicklung durch exogene Noxen über eine Interferenz mit der Laminin-β1-Integrin Wechselwirkung ein neuer, bisher nicht beschriebener Mechanismus.

3.3.4 Toxikologische Bewertung von PBDE und Flavonoiden

Die Herstellung und Verwendung der PBDEs ist aufgrund der Bioakkumulation in der Umwelt und der aktuellen Exposition des Menschen inzwischen durch regulatorische Maßnahmen in der EU eingeschränkt. In den USA folgte der größte Hersteller, Great Lakes Chemical Corporation, mit einem freiwilligen Verzicht seit Ende 2004 (Birnbaum and Cohen Hubal 2006). Trotz der Limitierung des Gebrauchs ist aufgrund des persistenten Charakters der PBDE erst in einigen Jahrzehnten mit einer Abnahme der Konzentrationen in Mensch und Umwelt zu rechnen. Zumal ein weiterer Eintrag durch ältere flammgeschützte Produkte kontinuierlich stattfindet.

Chlorierte organische Verbindungen, wie die polychlorierten Biphenyle (PCB), sind seit Jahren als umwelt- und gesundheitsschädigend im Bewußtsein der Öffentlichkeit verankert. Die bromierten Verbindungen sind chemisch eng mit diesen verwandt. Dass sie auch ein ähnliches Wirkpotential besitzen, zeigen die in dieser Dissertation vorgestellten Daten. Auch PCB wirken als endokrine Disruptoren der zellulären Schilddrüsenhormonhomöostase und stören die Calciumhomöostase (Mariussen and Fonnum 2006;Zoeller and Crofton 2000). In einem früheren Forschungsprojekt konnten wir bereits zeigen, dass die Aktivierung der

Schilddrüsenhormonrezeptoraktivität durch PCB zu Störungen der neuralen Differenzierung führt (Fritsche et al. 2005). Was das ENT Potential für den Menschen angeht, gibt es nach wie vor noch deutliche Wissenslücken. Die vorliegende Dissertation hat gezeigt, dass PBDE in einem humanen Zellsystem sowohl als endokrine Disruptoren des Thyroidhormonsignalwegs als auch als Modulatoren der intrazellulären Calciumkonzentration wirken können. Nun sind vergleichende Untersuchungen in entsprechenden Nagerzellsystemen notwendig, um diese gewonnenen *in vitro* Humandaten zu den vorhandenen Studien am Tier in Beziehung zu setzen. Gegebenenfalls wird eine toxikologische Neubewertung dieser Stoffgruppe in Hinblick auf ihr entwicklungsneurotoxisches Potential notwendig.

Für Grünteecatchine als Nahrungsergänzungsmittel gibt es bisher keine Abschätzung des toxischen Potentials für den Menschen. Dies resultiert hauptsächlich aus den multiplen Beobachtungen, dass Flavonoide die Entstehung von Tumoren und degenerativen Erkrankungen hemmen können. Viele an der Entstehung und Ausbreitung beteiligte zelluläre Signalwege sind jedoch auch für Entwicklungsprozesse essentiell. Da Grünteecatechine mit einer Vielzahl solcher Signalwege interagieren, ist es von großer Wichtigkeit, das Gefährdungspotential dieser Substanzen für Entwicklungsprozesse des Menschen zu untersuchen.
Unsere Ergebnisse zum Einfluss von Grünteeflavonoiden auf neurale Progenitorzellen des Menschen zeigen, dass einige von ihnen über die Blockierung der Interaktion zwischen β1-Integrin und Laminin die Adhäsion dieser Zellen *in vitro* hemmen. Weiterhin ist durch die gestörte Adhäsion auch die Migration der Progenitorzellen aus der Neurosphäre heraus inhibiert. Da die Adhäsions-/Migrationshemmung schon bei einer EGCG-Konzentration ab 2 µM zu beobachten ist und bei Probanden, die hoch konzentriertes EGCG in Form von Nahrungsergänzungsmitteln zu sich nahmen, Plasmakonzentrationen von bis zu 7,5 µM gemessen worden sind (Ullmann et al. 2003), ist eine entwicklungsneurotoxische Wirkung von Grünteeflavonoiden beim Menschen nicht ausgeschlossen. Um die Datenlage mit *in vivo* Resultaten zu untermauern, wurde im Rahmen der vorliegenden Dissertation auch eine tierexperimentelle Studie durchgeführt, die klären soll, ob hohe Konzentrationen von EGCG im maternalen Plasma negative Auswirkungen auf die fetale Gehirnentwicklung bei Ratten haben. Die Auswertung der Daten dieses Experiments stehen noch aus. Ohne solche Studien, welche den Metabolismus sowie die Verteilung des EGCG berücksichtigen, ist eine Risikoabschätzung von EGCG zum momentanen Zeitpunkt noch nicht möglich.

3.4 Humane Neurosphären als *in vitro* Alternativmodell zur Untersuchung von entwicklungsneurotoxischen Substanzen

Das Neurosphären-Zellsystem ist ein dreidimensionales auf humanen neuralen Progenitorzellen beruhendes Zellmodell. Voraussetzung für die Nutzung als *in vitro* Alternativmodell zur Untersuchung von entwicklungsneurotoxischen Substanzen sind vor allem drei Faktoren:

(i) eine exakte zellbiologische Charakterisierung des Modellsystems,

(ii) die Existenz von prädikativen reproduzierbaren Assays zur Bestimmung multipler Endpunkte,

(iii) die Validierung des Testsystems mit bekannten entwicklungsneurotoxischen Substanzen.

Die Arbeiten im Rahmen der vorliegenden Dissertation konnten wesentlich zu diesen Aufgaben beitragen.

Wir konnten demonstrieren, dass die Zellen innerhalb der Neurosphäre und während der Differenzierung in der Migrationsfläche den Progenitorzellmarker Nestin und Marker für die drei dominanten Zellltypen des zentralen Nervensystems GFAP (Astrocyten), β-III-Tubulin (Neuronen) und O4 (Oligodendrozyten) exprimieren (Fritsche et al. 2005;Moors et al. 2009). Auch im sich entwickelndem Gehirn sind diese Zelltypen durch eine Überlappung der einzelnen Reifungsprozesse koexistent (Andersen 2003;Rice and Barone S Jr 2000), was die physiologische Nähe des hNPC Zellmodells zur *in vivo* Situation unterstreicht. Besonders Gliazellen und Neurone bilden vielfach funktionelle Einheiten im Gehirn. So sezernieren Astrozyten neuroaktive Transmitter, wie Glutamat, Neuropeptide oder Steroide, die die Anzahl an Synapsen, den Vernetzungsgrad von Neuronen und auch die synaptische Aktivität erhöhen. Auch die Differenzierung von adulten Stammzellen in Neurone wird durch Astrozyten induziert (Gomes et al. 2001;Nedergaard et al. 2003;Svendsen 2002). Ebenso konnte gezeigt werden, dass Astrozyten protektiv für Neuronen sind (Giordano et al. 2009;Mahesh et al. 2006;Morken et al. 2005). Daraus ergibt sich, dass ein möglichst „physiologisches“ *in vitro* Modell für die Entwicklung des Gehirns idealerweise aus einer Kokultur neuraler Zellen bestehen sollte.
Mit zunehmender Differenzierungszeit nimmt der Anteil der unreifen nestin-positiven Zellen in der Kultur ab, während gleichzeitig die Zahl der GFAP-, ß(III)Tubulin- und O4-positiven

Zellen zunimmt (Moors et al. 2009). Darüber hinaus konnten Western Blot Analysen zeigten, dass die Expression von Synapsin 1 und der Dopa-Decarboxylase in differenzierenden Zellen über die Zeit von 4 Wochen ansteigt (Moors Dissertation 2007). Dies sind klare Zeichen für eine zunehmende Reifung innerhalb des Neurosphären-Systems und implizieren eine Eignung des Neurosphären-Systems nicht nur für entwicklungsneurotoxische, sondern auch für neurotoxische Fragestellungen.

Unter proliferierenden Bedingungen sind undifferenzierte Zellpopulationen am Rande der Neurosphären aufzufinden, während sich die Differenzierungsmarker exprimierenden Zellen in der Sphärenmitte befinden (Moors et al. 2009). Diese dreidimensionale Struktur von Neurosphären wird wahrscheinlich durch einen Wachstumsfaktorgradienten hervorgerufen und zeigt, dass sich die Zellen in der Sphäre selbst organisieren. Diese Eigenschaft bietet einen klaren Vorteil gegenüber der konventionellen „Monolayer-Zellkultur".

Nach dem Entzug von Wachstumsfaktoren und in Gegenwart einer extrazellulären Matrix migrieren die hNPCs aus der Neurosphäre in einer Form einer kreisrunden Migrationsfläche heraus. Dieser Prozess spiegelt *in vitro* die radiale Migration während der Gehirnentwicklung wieder (Baumann and Pham-Dinh 2001). Dabei ist die Migration von humanen NPCs abhängig von der extrazellulären Matrix. So migrieren humane NPCs nicht auf Kollagen, Fibronectin oder PDL alleine, jedoch auf Laminin, dem vorherrschenden Protein der extrazellulären Matrix in der subventrikulären Zone (Lathia et al. 2007;Tzu and Marinkovich 2008). Migration und Adhäsion sind dabei vom β1-Integrin abhängig (Gassmann et al. 2009, in Vorbereitung). Dass die neurale Entwicklung auch *in vivo* β1-Integrin abhängig ist, konnte Graus-Porta et al. (2001) anhand von Mäusen mit einem β1-Integrin Knockout in allen Neuronen und Gliazellen demonstrieren. Die Mäuse ohne β1-Integrin wiesen starke Fehlbildungen in der Gehirnstruktur auf und waren nach der Geburt nicht überlebensfähig.

In einem früheren Forschungsprojekt zeigte unsere Arbeitsgruppe, dass humane NPC auch eine Vielzahl an Neuronen-spezifischen Markern exprimieren. Es wurden Neurotransmitter-synthetisierende Enzyme, wie die Cholinacetyltransferase (ChAT), die Glutaminsäuredecarboxylase (GAD), die Dopa-Decarboxylase und auch die Tryptophanhydroxylase 1 (TPH-1) nachgewiesen, die auf eine Synthese der Neurotransmitter Acetylcholin, GABA, Dopamin und Serotonin hindeuten. Auch die Expression verschiedener neuronaler Rezeptoren konnte detektiert werden, die auf die Präsenz von NMDA-Rezeptoren, GABA-, dopamin- und acetylcholinerger Neurone hindeuten (Moors Dissertation 2007). Die im Rahmen dieser Arbeit durchgeführte elektrophysiologische Charakterisierung der NPC liefert weitere Hinweise auf den Reifegrad und die Funktionalität der Neuronen und Astrozyten. Sowohl die Applikation von unspezifischen Stimuli wie ATP als auch von spezifischen „Triggern" wie Acetylcholin und Glutamat führten zu einem Anstieg der intrazellulären Calciumkonzentration. Der Anteil der

reagierenden Zellen war dabei im Falle von Glutamat von der Differenzierungszeit abhängig, was vermuten lässt, dass ein gewisser Differenzierungsgrad der Zellen zur Reaktion auf diesen Stimulus Voraussetzung ist. Die Ergebnisse dieser Calcium-Imaging Versuche demonstrieren die Funktionalität cholinerger und glutamaterger Rezeptoren. Da verschiedene Noxen spezifisch auf bestimmte Neuronenpopulationen wirken, ist die Existenz verschiedener Neuronenpopulationen in humanen NPCs von besonderer Bedeutung für die Anwendung als entwicklungsneurotoxikologisches Modell.

Neben der Tatsache, dass ein *in vitro* Alternativmodell zur Untersuchung entwicklungsneurotoxischer Substanzen gut zellbiologisch charakterisiert sein muss und klare Parallelen zur *in vivo* Situation aufweisen sollte, ist es auch essentiell, dass etablierte Endpunkte messbar sind. „Assays“ zur Messung dieser Endpunkte sollten möglichst für Hochdurchsatzverfahren geeignet sein und Endpunkt-spezifische Kontrollen beinhalten (Breier et al. 2009). Im Rahmen dieser Dissertation wurde einerseits die schon existierenden Migrations-, Differenzierungs-, Viabilitäts- und Apoptoseassays optimiert und erweitert, andererseits neue Testmethoden für die Endpunkte Proliferation und Adhäsion etabliert (Moors et al. 2009; Gassmann et al. 2009 in Vorbereitung).

Der dritte wichtige Punkt bei der Entwicklung eines alternativen *in vitro* Testmodells ist die Validierung des Testsystems mit beschriebenen entwicklungsneurotoxischen Substanzen. Es hat sich gezeigt, dass das Neurosphären-Modell in der Lage ist, das entwicklungsneurotoxische Potential von Chemikalien, welche die Gehirnentwicklung nachweislich beim Menschen stören, zu identifizieren (Fritsche et al. 2005;Moors et al. 2007;Moors et al. 2009). Zu diesen Chemikalien gehören Quecksilberverbindungen, Alkohol und PCB (Grandjean and Landrigan 2006). Die Effekte dieser Substanzen auf Migration und Differenzierung scheinen dabei, mit den *in vivo* Daten überein zu stimmen. Darüber hinaus konnten die Ergebnisse dieser Dissertation Hinweise darauf geben, dass PBDE *in vitro* über ähnliche zelluläre Zielstrukturen wie PCB wirken.
Ein weiterer Vorteil unseres Neurosphären-Modells besteht darin, dass wir durch die Präparation von murinen NPC direkte Speziesvergleiche in Bezug auf die Suszeptibiltät gegenüber den erwähnten Substanzen durchführen können. Dieses Verfahren wurde im Rahmen dieser Dissertation erfolgreich für die Erfassung der Mechanismen, die zum entwicklungsneurotoxischen Potential von PAHs beitragen, angewandt (Gassmann et al. 2009, eingereicht). Diese Daten unterstreichen, dass ein auf humanen Zellen basierendes *in vitro* Modell einem auf Tierzellen beruhendem Testsystem weit überlegen ist, wenn es darum geht, ein Gefährdungspotential für den Menschen abzuschätzen. Um eine Validierung des Neurosphärensystems voranzutreiben, werden zurzeit innerhalb eines BMBF-

Verbundprojekts eine ganze Batterie von positiven und negativen Testsubstanzen auf ihr entwicklungsneurotoxisches Potential hin getestet.

Obwohl das Neurosphären-Modellsystem zur Testung von ENT durch viele Vorteile besticht, gibt es auch einige Nachteile, die im Folgenden erläutert werden.
Der Einsatz von Neurosphären zur Charakterisierung molekularer Mechanismen ist durch die Heterogenität der Sphären nur eingeschränkt möglich, da die Ergebnisse zellbiologischer Endpunkte in der Regel nicht auf einen spezifischen Zelltyp zurückgeführt werden können. Zudem ist die Anwendung moderner molekularbiologischer Methoden wie Transfektion in solchen dreidimensionalen Zellmodellen mit Schwierigkeiten verbunden. Auch die Frage der Reproduzierbarkeit der Ergebnisse in verschiedenen Individuen ist noch nicht vollständig geklärt, wobei dieses Problem bei der Verwendung von Zelllinien gleichermaßen existiert und in der Literatur in der Vergangenheit kaum berücksichtigt wurde.
Erste Patch-Clamp Untersuchungen konnten sowohl Einwärts- als auch Auswärtsströme identifizieren, aber in keiner der gemessenen Zellen konnte ein Aktionspotential ausgelöst werden, was auf einen geringen neuronalen Reifungsgrad des Systems rückschließen lässt (Gassmann et al. 2009, in Vorbereitung). Dies stellt eine Limitierung des Systems in Bezug auf die Testung von Substanzen, die einen Effekt auf elektrochemische Eigenschaften von Neuronen haben und z.B. Erregbarkeit von Nervenzellen beeinflussen, dar. Die weitere Ausreifung des Neurosphärensystems durch Zugabe bestimmter Faktoren zu forcieren ist Gegenstand laufender Projekte. Schlussendlich werden momentan die Testmethoden überwiegend manuell unter relativ großem Zeitaufwand durchgeführt, aber erste Verfahren vor allem zur Automatisierung der Bildauswertung und das parallele Messen mehrerer Endpunkte in einem Versuchansatz („Multiplexing“) werden schon erfolgreich angewandt. In nächster Zukunft steht die Umstellung möglichst vieler Assays auf das 96-well Format, das eine teilweise Automatisierung zulassen würde, im Focus der Arbeiten. Wir gehen davon aus, dass mithilfe dieser Strategien zumindest ein mittlerer Probendurchsatz mit dem Neurosphären-Testsystem realisiert werden kann.
Zusammenfassend zeigt diese Arbeit, dass das Neurosphären-Zellsystem ein viel versprechendes *in vitro* Modellsystem zur Bewertung von Entwicklungsneurotoxizität ist, das klare Vorteile gegenüber anderen momentan verfügbaren Testmethoden aufweist.

4. Zusammenfassung

Der Arylhydrocarbon Rezeptor (AhR) ist ein Liganden-aktivierter Transkriptionsfaktor, welcher unter anderem die Expression fremdstoffmetabolisierender Enzyme reguliert. Darüber hinaus spielt der AhR aber auch eine Rolle in der physiologischen Regulation von Proliferation, Apoptose und Zelldifferenzierung. Verschiedene Untersuchungen an Invertebraten, Vertebraten und auch Säugern weisen darauf hin, dass sowohl das Fehlen des AhR als auch eine unphysiologische Aktivierung durch exogene Liganden wie polyaromatische und polyhalogenierte Kohlenwasserstoffe entwicklungsneurotoxisch wirken können. Für den Menschen gibt es bisher kaum Daten zur physiologischen Rolle des AhR besonders in Bezug auf die neurale Entwicklung. Ziel der vorliegenden Dissertation war es daher, die Auswirkungen verschiedenster AhR-Liganden, die als Verunreinigung in Lebensmitteln oder bewusst als Nahrungsergänzungsmittel aufgenommen werden, auf die humane neurale Entwicklung *in vitro* zu untersuchen. Als Zellmodellsystem wurden normale neurale Progenitorzellen (NPCs) humanen Ursprungs verwendet, die als Neurosphären kultiviert wurden. Als Kontrollsystem zu den aus Tierversuchsstudien erhobenen Ergebnissen bezogen wir murine Neurosphären in die Untersuchungen mit ein.

Die Ergebnisse der vorliegenden Dissertation zeigen, dass die AhR-Agonisten 3-MC und B(a)p sowie der AhR-Antagonist MNF keine Wirkung auf die Proliferation und Migration humaner NPCs haben. Im Gegensatz dazu stören diese Substanzen solche Prozesse in murinen NPCs, was die Entwicklungsneurotoxizität dieser Substanzen bei Nagern *in vivo* wiederspiegelt. In dieser Arbeit wurde gezeigt, dass die beobachteten Spezies-spezifischen Unterschiede auf einer deutlich niedrigeren mRNS-Expression der AhR-Signalwegkomponenten in humanen im Vergleich zu murinen NPCs beruhen. Dies weist darauf hin, dass der Mensch deutlich unempfindlicher für AhR-vermittelte Entwicklungsneurotoxizität zu sein scheint als Nager.

Auch PBDE sowie die als natürliche AhR-Liganden ausgewählten Flavonoide interferieren mit der humanen neuralen Progenitorzellentwicklung. PBDE und Flavonoide inhibieren beide die Migration und PBDE hemmen darüber hinaus auch die Differenzierung der Zellen. Durch die zuvor beschriebene Beobachtung, dass der AhR in der humanen neuralen Progenitorzellentwicklung keine Rolle spielt, kann geschlossen werden, dass die nach PBDE- und Flavonoidexposition beobachteten Veränderungen nicht auf eine Aktivierung oder Inhibierung des AhR zurückgeführt werden können, sondern alternative Mechanismen in Betracht gezogen werden müssen. Weiterführende Untersuchungen zeigten, dass an der Entwicklungsneurotoxizität dieser Substanzen endokrine Disruption der TH-

Signaltransduktion, Störungen der Calcium-Homöostase sowie der Integrin-EZM-Interaktion beteiligt sind.

Die Ergebnisse dieser Arbeit beschreiben die Gefährdungspotentiale der entsprechenden Substanzen und können somit möglicherweise zur Risikoabschätzung für den Menschen beitragen. Nach wie vor wird bei der Grenzwertsetzung von Nager-Experimenten auf den Menschen extrapoliert und Interspezies-Unterschiede werden zu wenig berücksichtigt. Daher sind solche, auf primären menschlichen Zellen basierende *in vitro* Systeme, wie die in dieser Arbeit angewandten Neurosphären, vielversprechende Modelle zur Unterstützung der Bewertung der Toxizität von Chemikalien für den Menschen.

5. Abstract

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor which regulates the expression of xenobiotic metabolizing enzymes. Moreover, the AhR contributes to regulation of proliferation, apoptosis and cell differentiation. Several studies in invertebrates, vertebrates and mammals indicate that a lack of AhR or its activation by polyaromatic and polyhalogenated compounds cause morphological abnormalities of brains or deficits in cognition and/or behaviour. The physiological function of the AhR in humans, especially for neural development, is so far unknown. As humans are exposed to AhR ligands as contaminants in food or through intake of food supplements, the aim of this study was to examine the effects of such ligands on human neural development *in vitro.* As a model system for human neural development we employed primary fetal human neural progenitor cells (hNPCs), which are cultured as neurospheres. For comparison to animal data, mouse neural progenitor cells (mNPCs) were incorporated in the study.

This work shows that in contrast to mNPCs, AhR agonism or antagonism has no effects on hNPC development. While AhR modulation attenuates mNPC proliferation and migration, hNPCs remain unaffected. These results with murine neurospheres thereby mirror developmentally neurotoxic effects of AhR ligands in rodents *in vivo*. The observed species-specific differences are due to non-functional AhR-signaling in human neurospheres based on very low gene expression of AhR signaling cascade members in hNPCs. These data indicate that humans are protected against AhR-dependent developmental neurotoxicity (DNT) at this developmental stage.

Moreover, PBDEs and flavonoids, as natural ligands of the AhR, interfere with human neural development *in vitro*. Both PBDE and flavonoids inhibit hNPC migration, and PBDEs additionally reduce cell differentiation. Although these compounds are known to bind to the AhR, non-functional AhR-signaling in human neurospheres rules out this mechanism of action. Instead, PBDEs exert their DNT potential by endocrine disruption of cellular thyroid hormone signalling and disturbance of intracellular calcium homeostasis, while certain flavonoids interfere with integrin binding to the extracellular matrix.

The results of this dissertation describe the hazard of the respective compounds for human brain development. Threshold limits for chemicals are still extrapolated from animal data and species-specific differences are insufficiently taken into account. Therefore, test systems based on primary human cells like our neurosphere model are a promising approach for the evaluation of toxicity of chemicals in humans and might in the future contribute to risk assessment.

6. Abkürzungsverzeichnis

AhR	Arylhydrocarbon Rezeptor
AhRR	AhR Repressor
AIP	AhR-interagierendes Protein
ARNT	AhR nuclear translocator
ATP	Adenosintriphosphat
B(a)P	benzo(a)pyrene
bHLH/PAS	basic Helix-Loop-Helix/Per-ARNT-Sim
CAM	Zelluläres Adhäsionsmolekül
ChAT	Cholinacetyltransferase
CYP	Cytochrom P450
DRE	dioxin responsive element
EC	Epicatechin
ECG	Epicatechingallat
EGC	Epigallocarechin
EGCG	Epigallocatechingallat
ENT	Entwicklungsneurotoxizität
ER	Endoplasmatisches Retikulum
ERK1/2	Extrazellulär-signalregulierte Kinasen 1+2
EZM	Extrazelluläre Matrix
GABA	Gamma-Amino-Butter-Säure
GAD	Glutaminsäuredecarboxylase
GFAP	Glial Fibrillary Acidic Protein
GW	Gestationswoche
HAK	Halogenierte aromtische Kohlenwasserstoffe
Hsp90	Hitzeschockprotein 90
I3C	Indol-3-Carbinol
IP_3R	Inositol-1,4,5-triphosphat-Rezeptor
LD_{50}	Dosis, bei der 50% der Tiere sterben
MAPK	Mitogenaktivierte Proteinkinase
3-MC	3-Methylcholanthren
MNF	3'-Methoxy-4'-nitroflavon
NMDA	N-Methyl-D-Aspartat
NPCs	Neurale Progenitor Zellen

OECD	Organisation für wirtschaftliche Zusammenarbeit und Entwicklung
PAH/PAK	Polyzyklische aromatische Kohlenwasserstoffe
PAI	Plasminogen-Aktivator-Inhibitor
PBDE	Polybromierte Diphenylester
PC-12	Phäochromozytom-Zelllinie
PCBs	Polchlorierte Biphenyle
PCDF	Polychlorierte Dibenzofurane
PCR	Polymerase Kettenreaktion
PDL	Poly-D-Lysin
PKC	Protein Kinase C
REACH	Registrierung, Bewertung, Zulassung und Beschränkung chemischer Stoffe
RT	Reverse Transkription
RYR	Ryanodin-Rezeptor
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
THR	Thyroidhormon-Rezeptor
TPH-1	Tryptophanhydroxylase-1
TUNEL	TdT-mediated dUTP-biotin nick end labeling
XRE	Xenobiotic responsive element

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Danksagung

An dieser Stelle möchte ich mich bei allen Menschen bedanken, die meine Promotion ermöglicht haben:

Frau Prof. Ellen Fritsche möchte ich für die Bereitstellung des interessanten Themas und ihre umfassende Betreuung und Unterstützung danken. Ganz besonders möchte ich dabei hervorheben, dass sie mir nicht nur auf wissenschaftlicher Ebene immer mit Rat und Tat zur Seite stand, sondern mir auch viel vermittelt und ermöglicht hat, das zu meiner persönlichen Entwicklung beigetragen hat.

Bei Frau Prof. Christine R. Rose möchte ich mich für die fakultätsübergreifende Betreuung, durch die meine Dissertation erst möglich war, und die erfolgreiche Kooperation bedanken.

Ich danke dem „Chef“ der Toxikologie Prof. Abel und Dr. Klaus Unfried, die stets bereit waren, mir bei Fragen zu helfen und mich zu unterstützen.

Allen ehemaligen, alten und neuen Kollegen der Arbeitsgruppe danke ich für die gute Zusammenarbeit, die wertvollen Diskussionen/Hilfestellungen und ganz besonders für die entstandenen Freundschaften.

Mein besonderer Dank gilt meiner gesamten Familie und meinen Freunden, die mich immer tatkräftig unterstützt und in meinem Vorhaben bestärkt haben.

Eidesstaatliche Erklärung

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder einer ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 30.10.2009